Effect of contractile protein alterations on cardiac myofilament function in human heart failure

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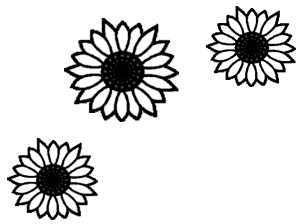

Effect of contractile protein alterations on cardiac myofilament function in human heart failure

Nadiya A. Narolska

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On the cover: Image of a single human cardiomyocyte exchanged with troponin complex containing $cTnI_{1\text{-}192}$, incubated with an antibody directed against C-terminus of cTnI.

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Effect of contractile protein alterations on cardiac myofilament function in human heart failure

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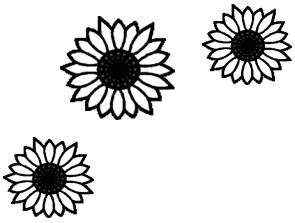

В пам´ять Тата,
для Мами і Лени

Contents

List of abbreviations

ACE	angiotensin-converting enzyme
AD(T)P	adenosine di(tri)phosphate
AF	atrial fibrillation
ALC	atrial myosin light chain
AU	arbitrary units
cAMP	cyclic adenosine monophosphate
cTn	cardiac troponin
cTn^{FL}	cardiac troponin complex containing full-length cTnI
cTn^{DEG}	cardiac troponin complex containing $cTnI_{1-192}$
cTnC	cardiac troponin C
cTnI	cardiac troponin I
$cTnI_{1-192}$	truncated cardiac troponin I containing aminoacid residues 1-192
cTnT	cardiac troponin T
DTT	dithiothreitol
EGTA	ethylene glycol-bis (amino-ethylether) N,N,N′,N′-tetra acetic acid
EC_{50}	median effective concentration
f_{app} (f)	rate constant of crossbridge attachment
F_{max}	maximal isometric force
F_{pass}	passive force
g_{app} (g)	rate constant of crossbridge detachment
HF	heart failure
IR	inhibitory region of troponin I
kDa	kilodalton
K_{ACT}	the rate of force rise
K_{TR}	rate of force redevelopment
$K_{REL,F}$	rate constant of the rapid phase of force relaxation
$K_{REL,S}$	rate constant of the slow phase of force relaxation
LV	left ventricle

LVEDP/ LVESP	left ventricular end-diastolic/systolic pressure
MHC	myosin heavy chain
MLC	myosin light chain
mRNA	messenger RNA
MyBPC	myosin binding protein C
n	number of observations
NADH	nicotineamide adenine dinucleotide
nH	steepness of the force-pCa or ATPase-pCa relation
NMR	nuclear magnetic resonance
NYHA	New York heart association
P_i	inorganic phosphate
pCa_{50}	mid-point of the force-pCa or ATPase-pCa relation
PKA	protein kinase A
ROS/RNS	reactive oxygen/nitrogen species
SEM	standard error of the mean
SERCA2a	sarcoplasmic reticulum Ca^{2+} ATPase 2a
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SR	sinus rhythm
TM	tropomyosin
t_{SLOW}	duration of the slow phase of force relaxation
UV	ultraviolet
V1	homodimer of α–myosin heavy chain
VLC	ventricular myosin light chain

Chapter 1

General introduction

The incidence of heart failure is increasing worldwide. At present heart failure is a major cardiovascular problem afflicting about 22 million individuals in the world. It is the reason for 20% of all hospital admissions among people above 65 years of age. According to the Netherlands Heart Foundation there are currently around 200 000 people diagnosed with heart failure in the Netherlands. About 10% of patients with heart failure die within 1 year, and about 50% die within 5 years after diagnosis.[1] Therefore, development of new therapeutical strategies for both prevention and treatment of this disease is of great importance.

Heart failure (HF) (or Congestive Heart Failure) is the complex of signs and symptoms that occurs when the heart fails to pump an adequate cardiac output to meet the metabolic demands of the body. To compensate for the decreased cardiac output, the end-diastolic volume of the failing heart increases. When the end-diastolic wall is stretched beyond the optimal length of its muscle fibres it composes, the ability of the heart to pomp the blood is reduced. This increases the venous and cardiac volume. Due to this progressive congestion of the veins and the heart with blood the expression a congestive heart failure is sometimes used. However, because not all patients have volume overload at the time of initial or subsequent evaluation, the term "heart failure" is preferred over the older term "congestive heart failure".

The syndrome of heart failure may occur as the end-point of a number of cardiovascular diseases, such as ischemic heart disease, hypertension, idiopathic cardiomyopathy, valvular defects and atrial fibrillation.[2,3] During the development of heart failure cardiac pump function declines.[4] Several potential mechanisms have been described to underlie this process (Figure 1.1).

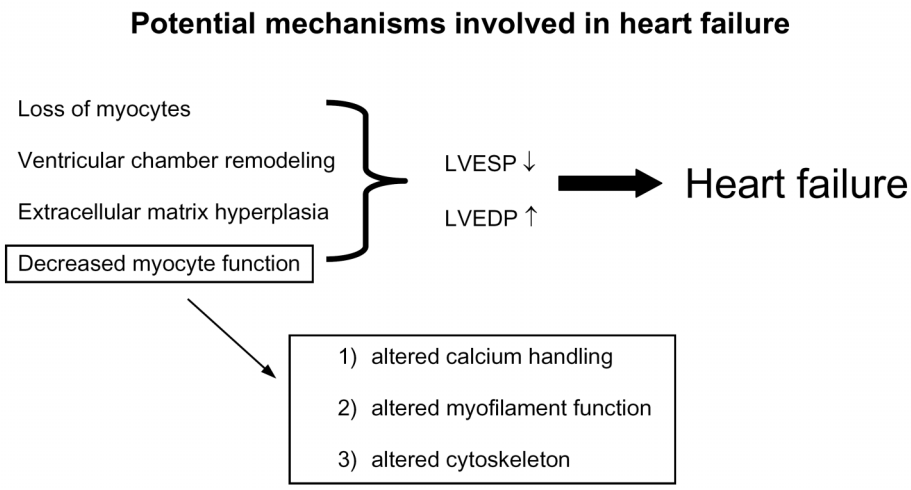

Figure 1.1. **Potential mechanisms involved in heart failure.** Abbreviations: LVESP and LVEDP denote left ventricular end-systolic and end-diastolic pressure, respectively. Adapted from[5].

End-stage heart failure often involves the general loss of functional myocytes, an increase of collagen content of the extracellular matrix (hyperplasia), ventricular chamber remodeling and decreased myocyte function. These processes lead to a gradual decrease in left ventricular end-systolic pressure (LVESP) and/or an increase in left ventricular end-diastolic pressure (LVEDP). Eventually, the decline in cardiac pump function results in the clinical syndrome of heart failure.[5]

It has been shown that in the end-stage failing human hearts, basal myocyte contractility is well preserved, while 'contractility reserve' (the ability to increase contractility with heart rate or sympathetic stimulation) is severely depressed.[6-8] These changes in muscle performance and regulation can explain the poor pump function, reduced exercise capacity and tachycardia intolerance (i.e. inability to endure high heart beating rate) characteristic for the failing human heart.[9] Depressed myocyte contractility is an important determinant of reduced pump function observed in heart failure.[9] However, most data from clinical trials indicate that inotropic therapy aimed to enhance cellular contractility of the failing heart increases rather than decreases mortality.[10] One explanation for the increased mortality is that this treatment, while increasing contractility, at the same time overstimulates signaling cascades (such as cAMP or calcium-calcineurin pathways)

that are already chronically activated. The current drug regimen consisting of β-blockers and ACE-inhibitors, which reduces neurohumoral stimulation of the heart proved to be beneficial,[11,12] however the underlying cellular mechanisms of their action are largely unknown yet. New targeted heart failure therapies that would increase or maintain cardiomyocyte contractility by selectively modulating specific contractile proteins are of particular interest. Hence, knowledge about the specific primary mechanisms involved in the development of heart failure in human tissue is essential for the development of new clinical interventions for heart failure.

At the molecular level, decreased cardiomyocyte function may be due to alterations in calcium handling, altered myofilament function, changes in the myocyte cytoskeleton, or a combination of these factors (Figure 1.1). The first evidence for a functional impairment at the myofilament level in failing human myocardium has been obtained more than 40 years ago.[13] Alpert and Gordon[13] demonstrated a markedly reduced myofibrillar ATPase activity in failing human hearts. Since then evidence for the involvement of myofilament protein alterations in heart failure has accumulated.[5,14-18] Changes in isoform composition, phosphorylation and degradation of both contractile and regulatory myofilament proteins and their functional consequences have been reported. However, the precise mechanism of myocyte dysfunction in heart failure remains largely unknown. In addition, the relative contribution of these alterations to *in vivo* cardiac pump function is not clear yet.

Cardiac myofilament contraction

Sarcomere structure

A schematic representation of the contractile apparatus of striated muscle is shown in Figure 1.2. The major proteins of the thick and thin filaments are indicated. The top panel reflects the sarcomeric structure during diastole, while the bottom panel reflects systole.

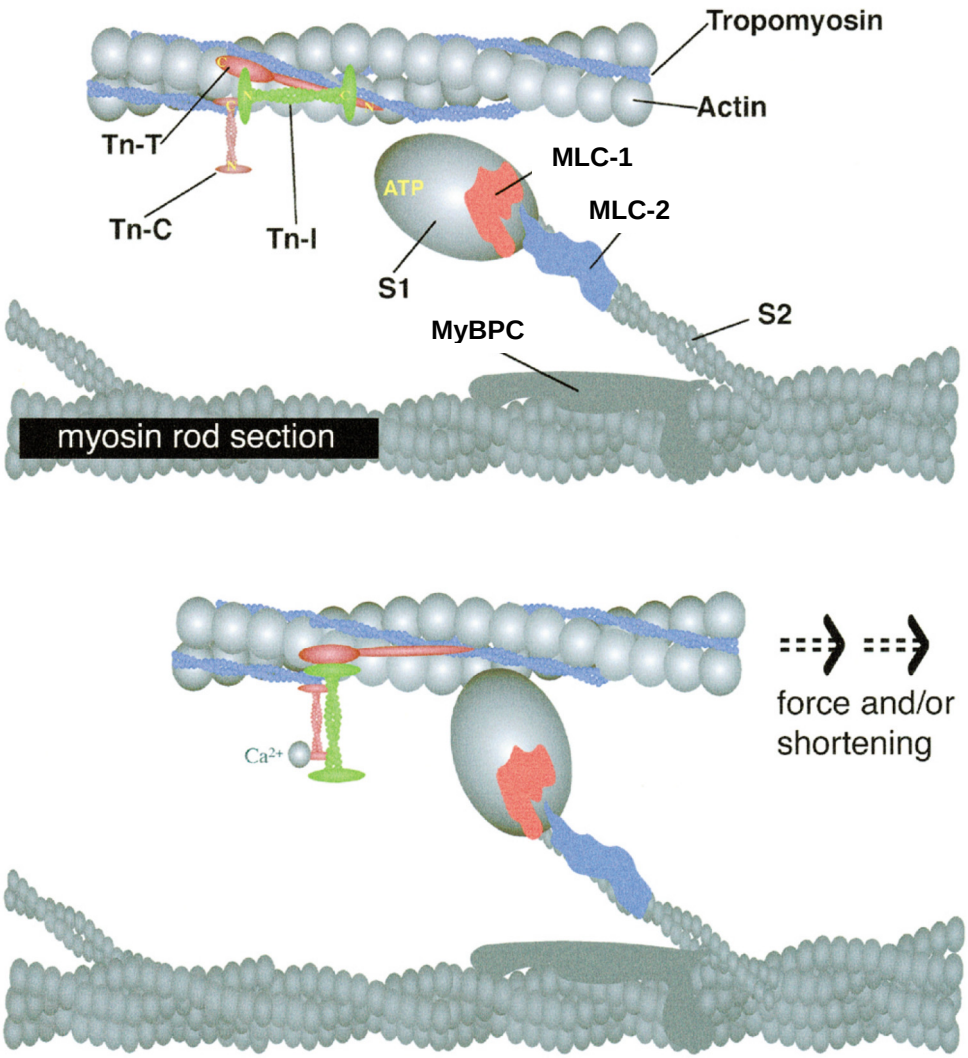

Figure 1.2. **Sarcomere structure.** The major contractile and regulatory proteins are shown in diastole (top panel) and systole (bottom panel). The thin filament is composed of actin, tropomyosin, and the troponin complex (Tn-T, Tn-C, and Tn-I). The thick filament is composed of myosin containing a globular head portion (S1), a hinged stalk region (S2) and a rod section. The S1 portion is associated with two light chains 1 and 2 (MLC-1; MLC-2). The myosin rod section also contains the myosin binding protein C (MyBPC). Adopted from[19].

The thick filament is a bipolar polymer of the motor protein myosin that interacts with actin to produce force and sarcomere shortening. Myosin is composed of two heavy chains with a molecular mass of ~200 kDa each and four light chains with molecular masses of ~20-24 kDa each. The *myosin heavy chains* (MHC) form a parallel coiled-coil structure with a large, globular region at the end, termed head or S1 (subfragment 1).[20] The myosin head is called a **crossbridge**, since this unit bridges the gap between the myosin and actin filaments in muscle. The site of the ATPase activity is located in the heads of the MHCs, which also carry the actin-binding site. Two different MHC isoforms are found in the human heart: the fast α- and the slow β-MHC. β-MHC predominates in the ventricles, while the atria contain a mixture of these two isoforms (β-MHC ranges from 0 to 29% of total MHC).[21] One pair of *myosin light chains* binds to each myosin head. The myosin light chains form two subfamilies, the essential light chains (MLC-1) and the regulatory light chains (MLC-2). Two different essential MLC isoforms exist, characteristic for atrial and ventricular tissue (ALC-1 and VLC-1, respectively) and at least three regulatory MLC isoforms are present in human heart (atrial ALC-2 and ventricular VLC-2 and VLC-2*). The regulatory MLC-2 can be phosphorylated by Ca^{2+} -calmodulin activated myosin light chain kinase and by protein kinase C.[22] The role of MLCs is to regulate force generating kinetics and thereby to tune cardiac function.[23] Other proteins have been shown to also play an important role in the thick filament structure and function. *Myosin binding protein C* (MyBPC) has a 1–7 myosin stoichiometry. It forms a collar around part of the myosin molecules. It modulates myocyte contraction, but is also involved in the assembling of the contractile filaments. The large elastic molecule *titin* plays a major structural role in the sarcomere contributing to passive and possible active elasticity as well as sarcomere stability. The *M-line proteins* (myomesin and M-protein) provide struts connecting the thick filaments together in the centre of the sarcomere.[24]

The main site for Ca^{2+} -regulation is the thin filament. The thin filament of cardiac muscle consists of actin, tropomyosin and the troponin complex (Figure 1.2).[17] *Actin* is a globular protein that, under physiological conditions, polymerises into elongated filaments of double helical strands. *Tropomyosin* (TM) an elongated protein that spans seven actin monomers, lies in the groove between actin strands and is polymerised head-to-tail along the actin filament. Human cardiac TM is a dimer of two identical α–helical chains, which are wound around each other in a coiled coil. Associated with each TM is *troponin* (cTn), a

globular protein complex consisting of three subunits: troponin C (cTnC), the Ca^{2+} binding protein; troponin I (cTnI), which inhibits the actin-myosin interaction and troponin T (cTnT), which transduces the Ca^{2+} binding signal to TM. Recently a large part of the atomic structure of human cTn using x-ray crystallographic analysis has been resolved (Figure 1.3).[25] In this atomic model, cTnI consists of 2 helixes H1 and H2, interacting with cTnT, a flexible inhibitory region (IR, residues 137-148), helixes H3 and H4, and a C-terminal domain (residues 192- 210). The IR and C-terminal domain are both essential for the inhibitory binding of cTnI to actin-tropomyosin in the absence of Ca^{2+}.

In diastole, crossbridges are either blocked from interacting with actin by TM or in a weak, rapid binding-unbinding state with actin without generating force (Figure 1.2). In systole, activation of the thin filament by Ca^{2+} ions allows strong binding crossbridge states, associated with force generation and ATP hydrolysis. It has been proposed[25] that the H3 helix of cTnI serves as a molecular switch, which at high Ca^{2+} concentration binds to cTnC (curled structure in Figure 1.3) and induces the detachment of IR and C-terminus from the actin filament, which through movement of TM allows the actin-myosin interactions.

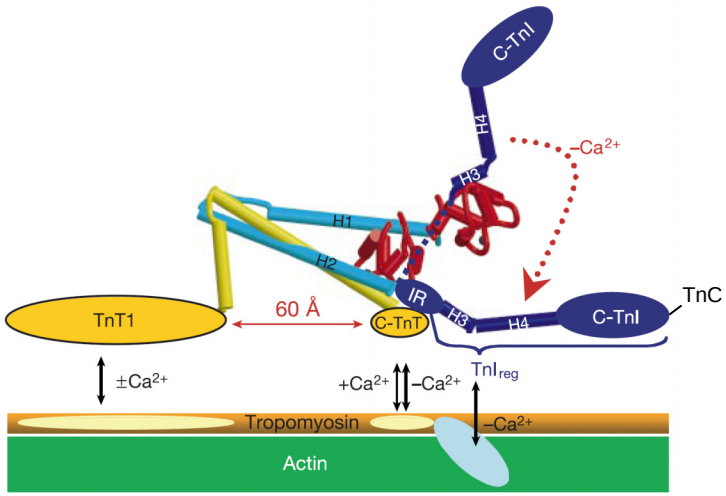

Figure 1.3. **A schematic representation of the interactions between troponin and other thin filament components based on x-ray crystallographic analysis.** The actin–tropomyosin-binding portions are schematically drawn: TnT1 and C-TnT are gray ellipsoids, and the inhibitory region (IR) and the C terminus of cTnI (C-TnI) are black ellipsoids. Light ellipsoids on the tropomyosin and actin represent sites of interaction with cTnT and cTnI. The solid arrows indicate the interactions between troponin and tropomyosin–actin.

Three state models

Biochemical[26] and structural[27] three-state models of thin filament regulation have been proposed. These biochemical and structural three-state models are comparable and provide complementary views of regulation, but are not necessarily equivalent. The data in this thesis are considered in the context of these models.

In the biochemical three state model,[26] tropomyosin can exist in equilibrium between blocked, closed and open states. These three states were termed analogous to terms describing an English shop: blocked (with a gate locked for overnight security), closed (with the door shut as at tea time or during lunch time), or open (for business). In the absence of Ca^{2+} ions, troponin stabilizes TM in a state that blocks any actin interaction with myosin (*blocked state*). In the presence of Ca^{2+}, the equilibrium shifts and TM adopts a state that allows myosin to bind relatively weakly to actin (*closed state*). In the presence of myosin, equilibrium shifts yet again and TM adopts a state that permits myosin to bind strongly to actin, to hydrolyse ATP, and to cooperatively propagate further binding of myosin heads to actin (*open state*).

In the structural three-state model[27] the Ca^{2+}-free troponin constrains TM in a position on actin that sterically blocks all, but weak myosin-binding sites. In the presence of Ca^{2+}, troponin allows TM to move on the surface of the actin filament, which exposes some amino acids on actin that interact strongly with myosin. Subsequent binding of myosin further moves TM around the actin filament, which exposes all strong myosin-binding sites on actin.

Brenner[28] has suggested that Ca^{2+} controls the transition between the weakly and strongly attached crossbridge states (i.e. from blocked to closed state). This transition can be described by an attachment rate constant, f_{app} (or f), and a detachment rate constant, g_{app} (or g).[28,29] The subscript *apparent* is used because both rate constants combine the rate constants of a number of steps in the actin-myosin ATPase cycle involving attachment of actin, various actin-myosin nucleotide states, and detachment. It has been found that g_{app} is independent of Ca^{2+} concentration, but f_{app} increases as a function of Ca^{2+}.

Alterations in myofilament function in heart failure

Alterations in myofilament function are attributed to changes in contractile proteins. In Figure 1.4 protein modification so far identified in HF are presented.

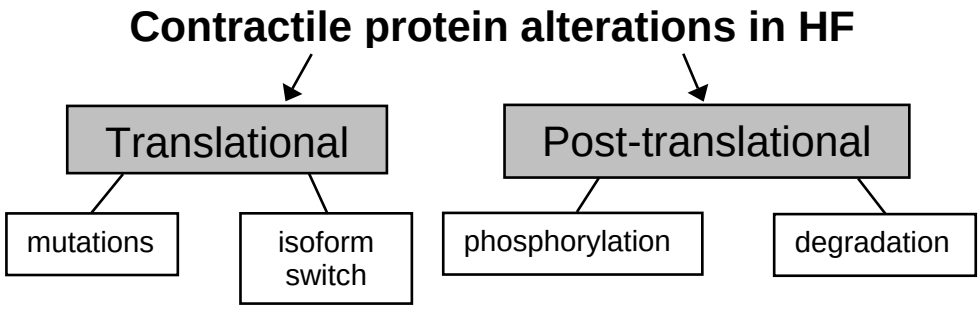

Figure 1.4. **Contractile protein alterations involved in heart failure.**

Translational modifications

Translational modifications are changes that occur in protein expression at the level of translation (transition from mRNA to polypeptide chain). Such changes include mutations and protein isoform shifts. *Mutations* are changes in the base sequence of DNA that alter genetic information. Familial hypertrophic and dilated cardiomyopathies are characterized by mutations in contractile proteins.[30] Over 300 dominant mutations in genes encoding sarcomere proteins were identified.[31] The mutations in β-MHC (~35%), MyBPC (~15%) and cTnT (~15%) have been reported to be the most frequent.[32] The severity and pattern of hypertrophy, age at onset of clinical manifestations, and progression to heart failure were found to be in part dependent on the precise sarcomere protein gene mutation. However, the pathophysiological mechanism how genetic mutations lead to functional defects are largely unknown. A putative cascade of events has been proposed[33] for development of familial hypertrophic cardiomyopathy: the mutation leads to a poison polypeptide that would be incorporated into the sarcomere. This would alter the sarcomeric function that would result (1) in an altered cardiac function (impaired or improved depending on mutation) and then (2) in the alteration of the sarcomeric and myocyte structure (hypertrophy). Whether this mechanism is common for all familial cardiomyopathies and how exactly the alterations in

sarcomere alter the phenotype and the function of the human heart still remains to be established.

Due to small differences in amino acid sequence, several forms of a protein may exist, which are called *isoforms*. In cardiac muscle different isoforms may be present of MHC, cTnT, MLC and titin. As indicated above in human myocardium the β-MHC isoform predominates in ventricles, whereas a variable mixture of α- and β-MHC is found in atria. During human heart failure the α-MHC fraction declines in both atria and ventricles.[21,34] The functional implications of this MHC switch in human myocardium are still unclear. In addition, a MLC isoform switch was reported in human heart failure. The appearance of ALC-1 in ventricles (up to 10%) has been associated with increased velocity of shortening and Ca^{2+}-sensitivity.[35,36] It has been suggested that this switch is an important component of compensated ventricular remodeling that may play a role in delaying or preventing progression to heart failure.[36] Also shifts from the adult cTnT to the less energy consuming fetal cTnT isoform[37] and from the stiff N2B to the more compliant N2BA titin[38] isoform were reported.

Post-translational modifications

Modifications of proteins, which occur after the translation process are called post-translational modifications. Such changes in contractile protein *phosphorylation* and *degradation* have been found in human heart failure.[14,17]

Cardiac troponin I is less phosphorylated in human end-stage failing hearts compared to donor hearts and this correlated with an increased responsiveness of the cardiac myofilaments to Ca^{2+}.[39-41] Also changes in MLC-2 phosphorylation[40] were observed in human tissue. The observed changes in contractile protein phosphorylation may be caused by a decreased activity of protein kinase A due to β-adrenergic desensitisation and receptor down-regulation and an increased activity of protein kinase C and protein phosphatases found in human heart failure.[42,43]

Degradation of thin filament proteins has been observed as a result of ischemic injury to the heart. The release of degraded as well as intact cTnI and cTnT into the blood is a well-established test for the occurrence of myocardial infarction within the clinical setting.[44] Cardiac TnI degradation was also found in viable cardiac tissue of patients with

ischemic cardiomyopathies[38,45,46] and has been associated with myocardial stunning, i.e. the reversible contractile dysfunction observed after abrupt periods of ischemia. The degradation of cytoskeleton proteins such as vinculin, desmin and α-actinin has been observed in infarcted human heart tissue as well.[47] Thus, it is possible that damage to cytoskeleton proteins could contribute to the diminished contractility of failing myocardium.[48]

Methodology

To answer the question about the relative role of contractile protein alterations in the onset and progression of heart failure several methodological approaches can be used. Employment of transgenic animal models provides a popular possibility to study the effects of a protein modification on *in vivo* function of the heart and evaluate its impact on morbidity and mortality. Models mimicking contractile protein changes such as e.g. cTnI degradation[45] and phosphorylation[49] have been developed. However, the question remains whether these transgenic studies can be extrapolated to contractile protein alterations in humans. It has been shown that some pathological mechanisms (e.g. stunning) in rodents are different than in large animals.[50] Besides, the results in transgenic mice models may be obscured by the species-specific development of compensatory mechanisms.[51]

In humans two different strategies can be followed to investigate the functional consequences of contractile protein alterations (Figure 1.5).

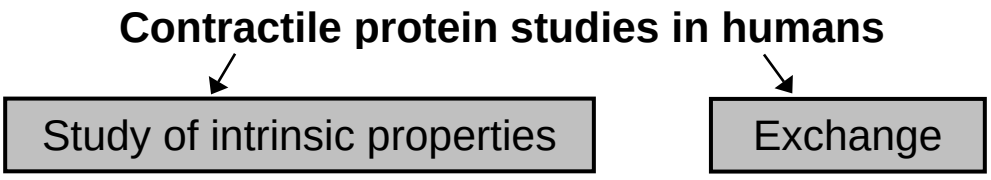

Figure 1.5. **Methodology of contractile protein studies in human myocardium.**

Study of intrinsic properties

The intrinsic variability in protein composition within human myocardium allows correlation of functional parameters with contractile protein composition. From these correlations the functional role of contractile protein alterations in heart failure can be derived. This methodology has been applied to study the role of MLC-2 and cTnI phosphorylation in human heart failure.[40] In the experiments described in this thesis this

approach was used to investigate the functional implications of the MHC isoform shift in heart failure. Using the variable expression of MHC isoforms in atrial and ventricular human tissue the functional characteristics of human α– and β–MHC isoforms were determined. To study the role of MHC isoform shift in diseased human myocardium, mechanical and energetic properties were investigated in atrial trabeculae from healthy patients and patients with atrial fibrillation and in thin ventricular muscle strips from non-failing donor and end-stage failing human hearts.

Exchange

This technique can be used to study the functional effects of an alteration in troponin subunit composition.[52,53] In this thesis, exchange experiments were performed for the first time in human cardiac tissue to study the specific effects of C-terminal degradation of cTnI. To this end, the human cardiac full-length cTnI and truncated cTnI ($cTnI_{1\text{-}192}$) peptides and other human troponin subunits (cTnT and cTnC) were expressed separately in *Escherichia coli* and purified using ion-exchange chromatography. The purified troponin subunits were reconstituted into the full troponin complex by mixing the subunits in a 1:1:1 molar ratio. The cardiac preparations were treated with an exchange solution containing an excess of troponin complex (*ca* 6 μM) with either full-length or truncated cTnI. In this way the troponin complex in the exchange solution kicks-off the endogenous complex and replaces it (Figure 1.6).

The exchange process mimics the natural process of cTn subunit replacement in cardiac myofilaments, where troponin exchanges into preexisting sarcomeres at random locations every 6-7 days (in rat cardiac tissue).[14] It has also been shown that exchange of the troponin complex is uniform and homogeneous along the myofilaments in cardiac myofibrils.[54] The advantage of this technique is that it allows to investigate the direct effect of cTn subunit alterations on contractility of human cardiac preparations without additional effects of changes in other contractile proteins found in failing human tissue or compensatory protein changes, which may develop in transgenic animals.

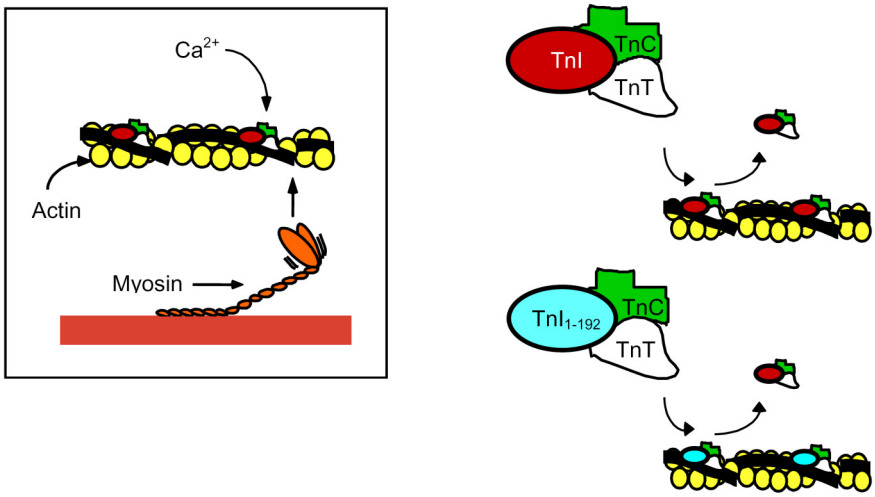

Figure 1.6. **Exchange of the full-length or truncated troponin complex in myofilaments.**

Aims of this study

The main objective of this thesis was to elucidate the effect of translational and post-translational alterations in contractile proteins occurring during heart failure on contractile function in human cardiac tissue. The shift of α- to β-MHC isoform was studied as an example of translational changes because this is a prominent feature in animal models of heart failure. The degradation of cTnI from its C-terminus was investigated as post-translation modification in failing myocardium, important during cardiac stunning and in remodelled non-infarcted heart. Isometric force and ATPase activity measurements were performed in skinned human cardiac trabeculae and muscle strips; force was measured in cardiac myofibrils and cardiomyocytes. The technique of troponin complex exchange was validated for human cardiomyocytes. To establish the link between altered protein composition and functional properties of cardiac tissue analysis of contractile protein composition was performed using one-dimensional SDS polyacrylamide gel electrophoresis and Western immunoblot analysis.

The questions addressed in this thesis are important to obtain insight into the cellular pathomechanisms of depressed contractility in human heart failure. This information will be important for the development of new targeted therapeutic interventions for the treatment of heart failure in the future.

The outline of this thesis is as follows:

In **Chapter 2**, the functional and morphological differences between human non-failing (donor) atrial and ventricular tissue were determined. In addition, it was investigated to what extent MHC isoform composition determines human myocardial performance using the variability present in MHC composition in atrial tissue. In **Chapter 3**, the functional properties and MHC isoform composition in both healthy and diseased ventricular and atrial human tissue were investigated to determine the functional relevance of relatively small changes in MHC isoform composition in failing human tissue. In **Chapter 4**, the functional consequences of C-terminal cTnI degradation in human cardiac myofibrils and cardiomyocytes were assessed. In **Chapter 5**, the changes in responsiveness to protein kinase A phosphorylation and to increasing sarcomere length of human cardiac myofilaments induced by cTnI proteolysis were studied to determine the effect of cTnI degradation on the β-adrenergic and preload-dependent responses. In **Chapter 6**, a summary of the major findings of the studies presented in this thesis and future research directions are presented.

Chapter 2

Myocardial contraction is 5-fold more economical in ventricular than in atrial human tissue

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Abstract

Cardiac energetics and performance depend on the expression level of the fast (α-) and slow (β-) myosin heavy chain (MHC) isoform. In ventricular tissue the β-MHC isoform predominates, whereas in atrial tissue a variable mixture of α- and β-MHC is found. In several cardiac diseases the slow isoform is upregulated, however, the functional implications of this transition in human myocardium are largely unknown. The aim of this study was to determine the relation between contractile properties and MHC isoform composition in healthy human myocardium using the diversity in atrial tissue. Isometric force production and ATP consumption were measured in chemically skinned atrial trabeculae and ventricular muscle strips, and rate of force redevelopment was studied using single cardiomyocytes. MHC isoform composition was determined by one-dimensional SDS-gel electrophoresis. Force development in ventricular tissue was about 5-fold more economical, but 9 times slower, than in atrial tissue. Significant linear correlations were found between MHC isoform composition, ATP consumption and rate of force redevelopment. These results clearly indicate that even a minor shift in MHC isoform expression has considerable impact on cardiac performance in human tissue.

Introduction

The contractile protein myosin consists of two heavy chains (MHC), which contain the actin- and ATP-binding sites, and two pairs of light chains (MLC). The motor function of myosin is performed by MHC, while the MLCs exert a regulatory role.[23,55,56] Two different isoforms of MHC (α and β) are expressed in mammalian cardiac muscle. Although they have 93% sequence homology,[57] animal studies indicated that these isoforms possess distinct functional properties.[58,59] Studies on rat and rabbit tissue indicated that the fast α-MHC isoform exhibits a two to three times higher actin-activated ATPase activity[58] and actin filament sliding velocity[59] than the slow β-MHC isoform.

In human myocardium the β-MHC predominates in ventricles, whereas a variable mixture of α- and β-MHC is found in atria. During human heart failure the α-MHC fraction declines in both atria and ventricles.[21,34] However, the functional implications of this MHC

switch in human hearts are still unclear. Moreover, little is known about differences in contractile features and energy consumption between atrial and ventricular tissues.

Thus, the first aim of the present study was to determine functional differences between human atrial and ventricular tissue. The second aim was to determine to what extent MHC protein composition determines human myocardial performance using the variability found in MHC composition in atrial tissue. Isometric force production, ATP utilisation and their Ca^{2+}-sensitivities were measured in chemically skinned atrial trabeculae and ventricular muscle strips from human hearts. The main advantage of skinned preparations is that they allow standardisation of the conditions (e.g. composition of intracellular milieu and sarcomere length) under which functional properties are studied. Moreover, simultaneous measurement of force and ATP consumption in skinned myocardial tissue allows unambiguous determination of the relation between contractile and energetic properties. The rate of force redevelopment was recorded in single Triton-skinned cardiomyocytes in order to obtain information on the kinetic properties of the actomyosin interaction. The MHC composition of the human atrial and ventricular biopsies was determined by one-dimensional gel electrophoresis and correlated with the functional properties measured in tissue from the same hearts.

Methods

Preparations

Trabeculae were isolated from right atrial appendages obtained during coronary bypass surgery on patients (n=14; age 41-80 years) with normal left ventricular function. Ventricular muscle strips were isolated from left ventricular biopsies, which were obtained from healthy donor hearts (n=6; age 23-54 years) that could not be transplanted due to technical reasons, and were stored in liquid nitrogen. Samples were obtained after informed consent and with approval of the local ethics committees. The investigation conforms with the principles outlined in the Declaration of Helsinki. 20 atrial trabeculae and 13 ventricular strips were isolated in cold relaxing solution (pH 7.0; in mmol/l: free Mg^{2+} 1, KCl 145, EGTA 2, ATP 4, imidazole 10) and then chemically skinned in relaxing solution with 1% Triton X-100 overnight (4°C). Ventricular muscle strips were cut longitudinally, i.e. in parallel to the long axis of the cardiomyocytes in order to minimize damage. Mean

dimensions (± S.E.M.) of the atrial and ventricular preparations amounted to 1.61±0.12 and 2.36±0.17 mm in length, 435±25 and 472±41 μm in width, and 384±22 and 495±34 μm in depth, respectively. Part of the biopsy was used for mechanical isolation of single cardiomyocytes as described previously.[40,60] Before mechanical isolation, tissue was defrosted in relaxing solution. During the isolation the tissue was kept on ice. Tissue samples were mechanically disrupted within a few seconds in relaxing solution using a small glass tissue homogeniser. Isolated myocytes were immersed for 5 minutes in relaxing solution containing 0.5% Triton X-100. To remove the Triton, cells were washed twice in relaxing solution. Thereafter, a single myocyte was attached between a force transducer and a piezoelectronic motor. The remainder of the biopsy was used for determination of MHC composition.

MHC composition

MHC composition was analysed by one-dimensional SDS-gel electrophoresis (1D SDS-PAGE), silver staining and laser scanning densitometry as described by van der Velden et al.[40,61] 1D SDS-PAGE was performed using an acrylamide to bis-acrylamide ratio of 200:1 in the separating gel (12% acrylamide; pH 9.3) and of 20:1 in the stacking gel (3.5% acrylamide; pH 6.8). Densitometric analysis was performed on a LKB UltroScan XL Enhanced Laser Densitometer (LKB Produkter AB, Bromma, Sweden) using the GelScan XL software package (Pharmacia, Uppsala, Sweden). To check for linearity, different amounts of atrial and ventricular tissue (0.2-1.0 μg) were loaded and the density of the MHC bands was analysed. Based on these determinations 0.5 μg was chosen for sample application as it was found to be within the linear range. In one ventricular sample, containing a relatively small amount of the α-MHC isoform (Figure 2.1C), the intensity profile was fit to the sum of 2 Gaussian curves representing the α- and β-isoforms and the ratio was calculated from the area underneath the α-peak divided by the total MHC area. For validation of MHC isoform analysis various mixtures (3:1, 1:1, 1:3) of an atrial (84.7% α-MHC) and a ventricular (99.3% β-MHC) tissue sample were applied. The percentage of β-MHC obtained from the densitometric analysis correlated well with the amount of ventricular sample present in the mixture (r^2=0.97, Figure 2.1A). Since the distance on gel between α- and β-MHC bands is increased by using less total acrylamide a comparison was

made between 8% SDS-PAGE[21,34] and 12% SDS-PAGE.[40,61] No significant differences were found between the data obtained with the two gel protocols.

Measurements of force, ATP consumption and rate of force redevelopment

The experimental procedures and equipment used were as described previously.[62,63] ATPase activity was measured by enzymatic coupling of ATP resynthesis to the oxidation of NADH, which could be quantified photometrically from the absorbance of near-UV light. The composition of relaxing, preactivating and activating solutions was calculated as described recently.[64] Isometric force and ATPase activity were measured at saturating and subsaturating Ca^{2+} concentrations at 20±1°C. Maximum force was determined when a steady-state force was reached. In a few ventricular preparations the force values reached after approximately 3 minutes of activation were used as maximal values. On average force decline between the first and the last maximal control activation amounted to 18±1 and 10±2% for atrial and ventricular preparations, respectively. The Ca^{2+}-activated ATPase activity was determined by subtraction of basal ATPase activity (measured in relaxing solution) from total ATPase activity measured in activating solution with various Ca^{2+} concentrations. The length of the preparations was adjusted on the basis of passive tension by stretching them to 1-2 kN/m^2. In two trabeculae it was possible to examine sarcomere length by means of laser diffraction. The sarcomere lengths found after adjustment of the length of the preparations amounted to 2.20 and 2.15 μm. This corresponds well with the values found by Kentish et al.[65] in rat trabeculae. The cross-sectional area of the preparations was estimated assuming an elliptical shape, i.e. cross-sectional area = (width × depth × π)/ 4.

The rate of force redevelopment was determined at saturating Ca^{2+} concentration in Triton-skinned single cardiomyocytes at 15±1°C.[63] In brief, when reaching a steady level of force the myocyte was rapidly shortened and after a delay of 30 ms, restretched by 20% of its length. Upon shortening force dropped to zero and upon restretch force redevelopment occurred to the initial steady level. Force redevelopment was fitted to a single exponential to estimate the rate of force redevelopment (K_{TR}).

Histochemical analysis

Histochemical analysis was performed on 6 atrial trabeculae and 5 ventricular preparations. At the end of the experiment preparations were embedded in relaxing solution containing 15% (w/v) gelatin and frozen in liquid nitrogen. Tissue sections (5 μm) were cut with a cryostat (Leica CM 1850, Rijswijk ZH, Netherlands) at -22°C. Sections were collected on slides treated with Vectabond (Vector Laboratories, Burlingame, CA), air dried for 20 min and slides were stored at –80°C. The sections were fixed at room temperature for 5 min in a 4% formalin in 0.1 M phosphate buffer, pH 7.4. After hematoxylin and eosin staining, the sections were dehydrated and mounted in Entallan (Merck, Darmstadt, Germany). Sections were studied as described previously[66] using a Leica DMRB microscope (Wetzlar, Germany). Images were collected with a Sony XC-77CE camera (Towada, Japan) connected to a LG-3 frame grabber (Scion, Frederick, MD, USA) on personal computer, and analysed using public domain software (NIH Image 1.61). The myofibrillar area and interstitial space were determined relative to the cross-sectional area and expressed as percentages.

Data analysis

Force-pCa and ATPase-pCa relations were fit by a non-linear fit procedure to a Hill equation:[67]

$$F(Ca^{2+})/F_0=[Ca^{2+}]^{nH}/(Ca_{50}{}^{nH}+[Ca^{2+}]^{nH}),$$

where F is steady state force (or ATPase activity). F_0 denotes the steady force (or ATPase activity) at saturating Ca^{2+} concentration, nH reflects the steepness of the relation, and Ca_{50} (or pCa_{50}) represents the midpoint of the relation.

All data were averaged per patient. Values are given as means ± S.E.M. of *n* patients. All statistical statements are based on two tailed Student's *t*-tests with a 0.05 level of significance.

Results

MHC composition

MHC isoforms were separated using 1D SDS-PAGE (Figure 2.1). Densitometric analysis of the SDS-gels (Figure 2.1C) revealed that atrial tissue consisted of a variable mixture of α- and β-MHC (β-MHC ranged from 6.9 to 51.3% of total MHC content), while β-MHC was the predominant isoform in ventricular tissue (Table 2.1). In two of the ventricular samples low expression levels of α-MHC were detected. Low abundance in these two samples was confirmed by Western immunoblotting. Western immunoblotting, performed on all ventricular samples using a specific monoclonal antibody against α-MHC,[40] yielded negative results except in these two samples.

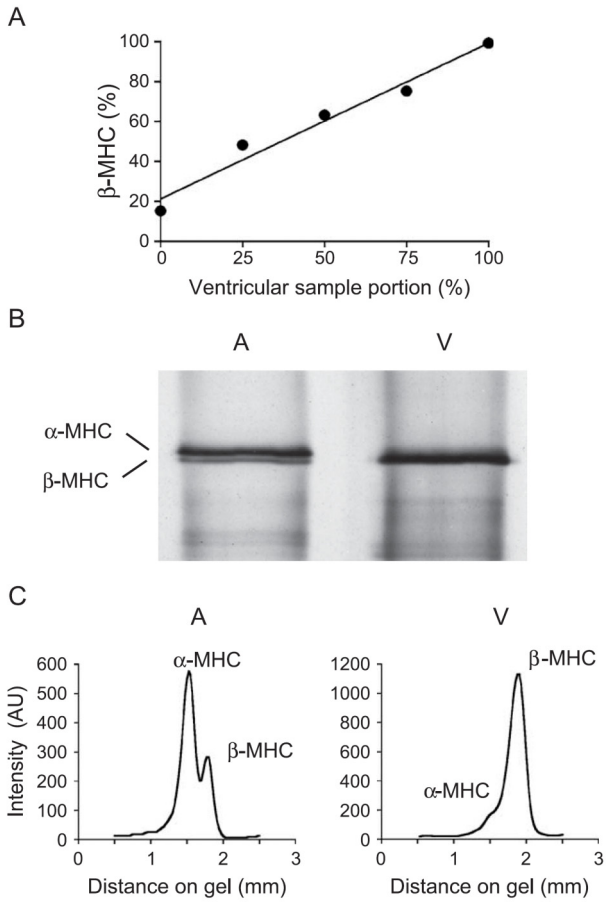

Figure 2.1. **MHC composition in atrial and ventricular tissue. A**. Validation of MHC analysis by SDS-PAGE using various mixtures (3:1, 1:1 and 1:3) of an atrial (84.7% α-MHC) and a ventricular (99.3% β-MHC) tissue sample. The percentage of β-MHC obtained from the densitometric analysis correlated well with the amount of ventricular sample present in the mixture. Regression line: β-MHC (in %) = (21.3±4.9) + (0.78±0.08) × ventricular tissue sample portion (in %) (r^2=0.97, P<0.05). **B.** Silver stained SDS-polyacrylamide gel of MHC proteins in an atrial (A) and a ventricular (V) tissue sample. **C.** Laser densitometric scan corresponding to the atrial (A) and ventricular (V) samples shown in Figure 2.1B. AU, arbitrary units.

***Table 2.1*. Mean parameters in atrial and ventricular tissue**

Parameter	Atrial (n=14)	Ventricular (n=6)
β-myosin heavy chain content (% of total MHC)	24.6±3.2	94.7±5.2*
Ca^{2+}-activated ATPase activity per preparation volume ($mmol \cdot l^{-1} \cdot s^{-1}$)	0.147±0.015	0.045±0.007*
Basal ATPase activity at pCa 9 (% of total)	11±1	11±1
Maximal tension ($kN \cdot m^{-2}$)	13.7±1.0	18.6±2.3*
pCa_{50} force	5.68±0.04	5.67±0.04
nH force	2.70±0.17	2.29±0.29
pCa_{50} ATPase activity	5.69±0.04	5.68±0.04
nH ATPase activity	2.33±0.14	2.36±0.35
Tension cost ($mmol \cdot kN^{-1} \cdot m^{-1} \cdot s^{-1}$)	11.4±1.4	2.4±0.3*
Maximal K_{TR} (s^{-1})	7.76±1.54	0.87±0.06*
Myofibrillar tissue (%)	50±9	89±2*
Interstitial space (%)	20±4	11±2
Maximal tension corrected for myofibrillar tissue ($kN \cdot m^{-2}$)	32.8±8.5	21.1±2.1
Ca^{2+}-activated ATPase activity per preparation volume corrected for myofibrillar tissue ($mmol \cdot l^{-1} \cdot s^{-1}$)	0.260±0.025	0.051±0.011*

Abbreviations: pCa = -log [Ca^{2+}]. pCa_{50}, Ca^{2+} concentration at which half of the maximal isometric force or ATPase activity is obtained; nH, steepness of pCa-force and pCa-ATPase activity relationships. Values are given as mean ± S.E.M. of *n* patients. Myofibrillar tissue and interstitial space were determined for 6 and 5 preparations of atrial and ventricular tissue, respectively. *significant at $P<0.05$, atrial versus ventricular.

Functional properties

Recordings of isometric force and ATP consumption in an atrial trabecula and a ventricular muscle strip during a contraction-relaxation cycle at saturating Ca^{2+} concentration are shown in Figure 2.2A and B. The slope of the regression line fitted to the NADH absorbance signal (Figure 2.2B) was considerably steeper in atrial than in ventricular

preparations. Indeed, the maximal rate of Ca^{2+} activated ATP consumption (i.e. ATPase activity per preparation volume) was on average 3.3 times larger in atrial tissue than in ventricular tissue (0.147±0.015 and 0.045±0.007 $mmol \cdot l^{-1} \cdot s^{-1}$, respectively). Maximal isometric tension (i.e. force divided by cross-sectional area) was lower in atrial trabeculae than in ventricular muscle strips. Table 2.1 provides mean values of Ca^{2+}-sensitivity (pCa_{50}) and steepness of the relationships (nH) of both force and ATP consumption. No significant difference was found in these parameters between atrial and ventricular preparations. Representative examples of force-pCa and ATPase activity-pCa curves obtained in one atrial trabecula are shown in Figure 2.3.

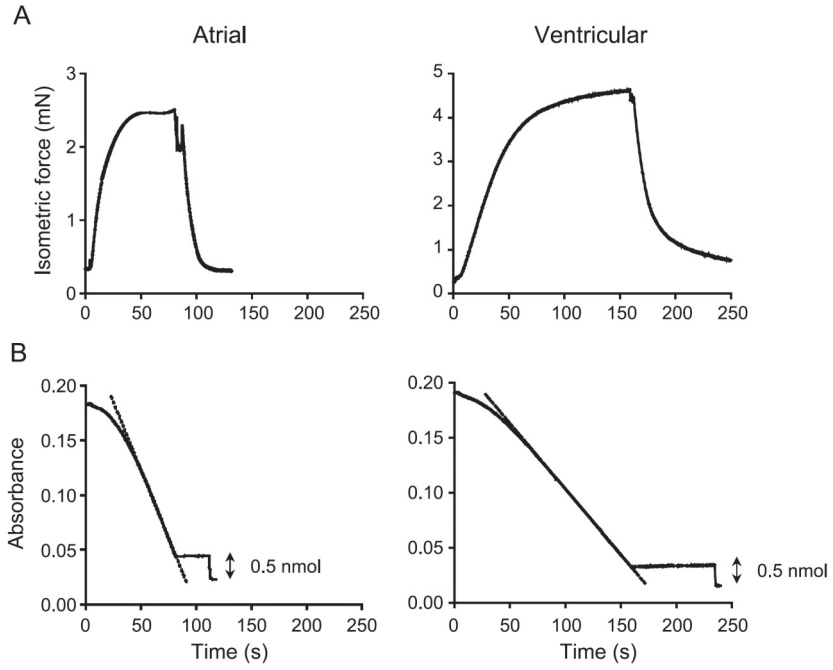

Figure 2.2. **Recordings of force and NADH absorbance in an atrial trabecula and a ventricular strip during a contraction-relaxation cycle. A.** Force production and **B.** NADH absorbance recorded at saturating Ca^{2+} concentration. When the preparation was transferred to the activating solution, isometric force developed and the NADH absorbance signal started to decline. ATP consumption was determined by calculation of the slope of the dotted regression line fitted to the absorbance signal during the last 20 seconds of activation. After returning trabeculae into the relaxing solution 0.5 nmol ADP was injected into the measuring bath to calibrate the absorbance signal. The zero level of the absorbance signal was arbitrarily chosen. Dimensions of the preparations were 2.5 mm in length, 420 µm in width and 380 µm in depth for atrial and 2.7 mm in length, 480 µm in width and 507 µm in depth for ventricular.

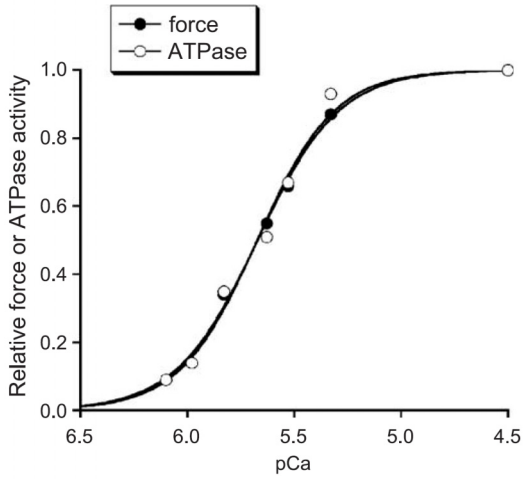

Figure 2.3. **Ca^{2+}-sensitivity of isometric force and ATPase activity.** The force-pCa and ATPase activity-pCa relation of an atrial trabecula from one atrial biopsy are shown as representative examples. Isometric force and ATP utilisation rate at submaximally activating Ca^{2+} concentrations are normalised to the control force/ATP utilization rate found at saturating Ca^{2+} concentration (pCa 4.5). Both the force-pCa and ATPase activity-pCa relations were fitted to a Hill equation.

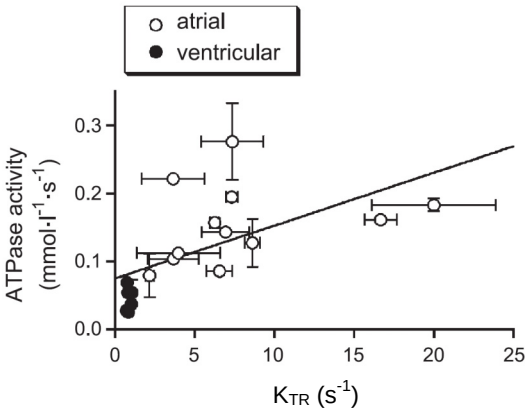

Figure 2.4. **Correlation between K_{TR} and ATPase activity.** A linear relationship exists between the rate of force redevelopment and mean ATPase activity (n=12 for atria and n=6 for ventricles). Regression line: ATPase activity/volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.07±0.02) + (0.008±0.003)× K_{TR} (in s^{-1}) (r^2=0.35, P<0.05).

Tension cost was calculated for each preparation by dividing ATP consumption per preparation volume by force per cross-sectional area. The preparation volume equals cross-sectional area multiplied by preparation length. Therefore, this measure has the advantage that it is independent of possible inaccuracy in the determination of cross-sectional area of the preparation. Tension cost is a measure of muscle economy, i.e. the rate of ATP splitting necessary for maintenance of isometric force. Tension cost (in $mmol \cdot kN^{-1} \cdot m^{-1} \cdot s^{-1}$) amounted to 11.4±1.4 in atrial tissue and to 2.4±0.3 in ventricular tissue. This implies that the economy of force maintenance in ventricular tissue is about 5 times larger than in atrial tissue.

The rate of force redevelopment (K_{TR}) measured in single isolated cardiomyocytes was on average 9 times higher in atrial than in ventricular tissue. These values are also summarized in Table 2.1. When ATPase activity was plotted as a function of K_{TR} (Figure 2.4) a weak but significant linear correlation was found between these two parameters (r^2=0.35; P<0.05).

Correlation between MHC content and functional properties

Significant linear correlations were found between maximal isometric force, rate of ATP consumption, tension cost and MHC composition (Figure 2.5). The rate of ATP consumption of preparations with pure (100%) α-MHC (estimated by extrapolation of the regression line) was approximately 5 times higher than the rate of preparations with pure β-MHC (0.181±0.018 and 0.039±0.014 $mmol \cdot l^{-1} \cdot s^{-1}$, respectively, for extrapolated values ± their standard errors). In addition, a significant negative correlation was found between mean K_{TR} and β-MHC content (r^2=0.55) (Figure 2.6). The K_{TR} associated with pure α-MHC expression calculated from the regression line (10.76 s^{-1}) was considerably higher than the values obtained in ventricular tissue (0.87 s^{-1}).

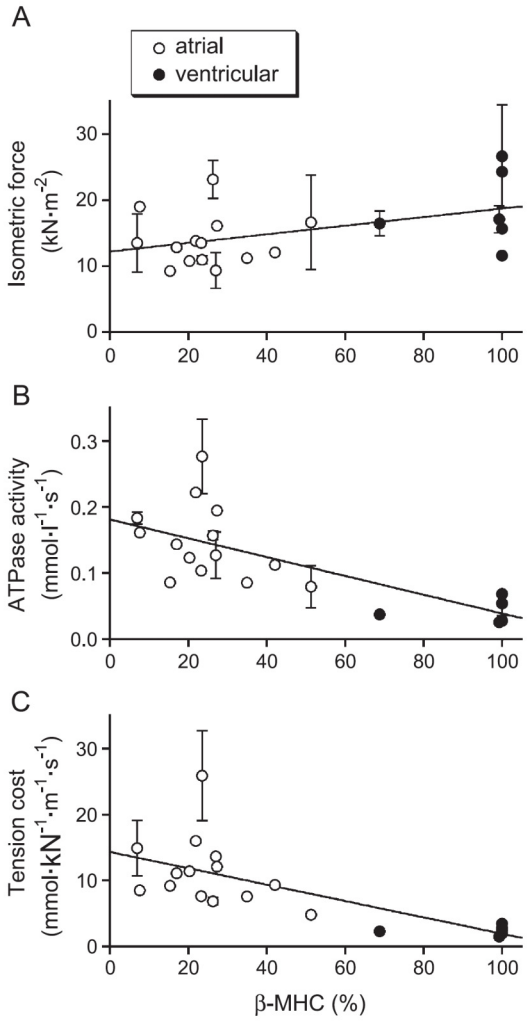

Figure 2.5. **Correlations between β-MHC content and isometric force, ATP consumption and tension cost. A.** Isometric force per cross-sectional area averaged per every patient (*n*=14 for atria and *n*=6 for ventricles) depended significantly on β-MHC composition. Regression line: isometric force/cross-sectional area (in $kN \cdot m^{-2}$) = (12.2±1.7) + (0.07±0.03)× β-MHC (in %) (r^2=0.22, P<0.05). **B.** A significant negative correlation exists between β-MHC fraction (% of total MHC) and mean ATPase activity for every patient (*n*=14 for atria and *n*=6 for ventricles). Regression line: ATPase activity/volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.18±0.02) – (0.0014±0.0003)× β-MHC (in %) (r^2=0.54, P<0.05). **C.** A significant correlation was also found between β-MHC content and mean tension cost for every patient (*n*=14 for atria and *n*=6 for ventricles). Regression line: Tension cost (in $mmol \cdot kN^{-1} \cdot m^{-1} \cdot s^{-1}$) = (14.32±1.62) – (0.124±0.028)× β-MHC (in %) (r^2=0.51, P<0.05).

Myofibrillar density

Histochemical analysis revealed that the average density of myofibrillar tissue was significantly lower in atrial (Figure 2.7A, C) than in ventricular (Figure 2.7B, D) preparations (50±9 and 89±2%, respectively, Table 2.1), mainly because in atrial tissue an outer layer of connective tissue was found and occasionally blood vessels were present (Figure 2.7A). The difference in the percentage of interstitial space in atrial (20±4%) and ventricular (11±2%) tissue did not reach statistical significance (P=0.09; Table 2.1).

The overall difference in amount of myofibrillar tissue between atrial and ventricular preparations may explain part of the differences in isometric force and ATP consumption between atrial and ventricular tissue. To investigate this point, data were corrected for the proportion of myofibrillar tissue. It abolished the difference in maximal tension, increased the difference in ATPase activity and slightly enhanced the relative difference in the extrapolated ATPase-values of the "pure" isoforms (Table 2.1). These results suggest that the difference in maximal isometric force found between atrial and ventricular preparations was due to the difference in the amount of myofibrillar tissue. Within the preparations used for histochemical analysis (n=11) no correlation was present between maximal force and MHC composition both before and after correction of the data for the proportion of myofibrillar tissue (Figure 2.8A). However, the correction slightly improved the correlation between ATPase activity and β-MHC composition as reflected by an increase in the r^2 value from 0.72 for the uncorrected data to 0.79 for the corrected data (Figure 2.8B), but, as expected, it did not influence the values obtained for tension cost.

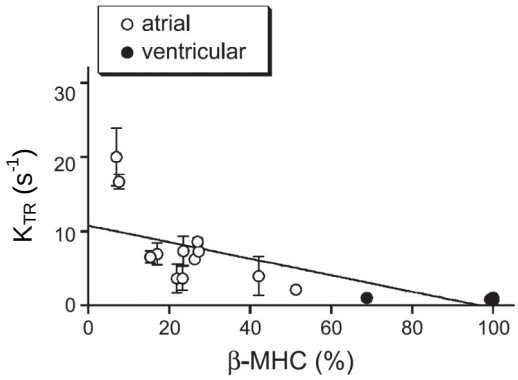

Figure 2.6. **Correlations between β-MHC content and rate of force redevelopment (K_{TR}).** A significant correlation exists between β-MHC content and mean K_{TR} for every patient (*n*=12 for atria and *n*=6 for ventricles). Regression line: K_{TR} (in s^{-1}) = (10.76±1.49) – (0.111±0.025)× β-MHC (in %) (r^2=0.55, P<0.05).

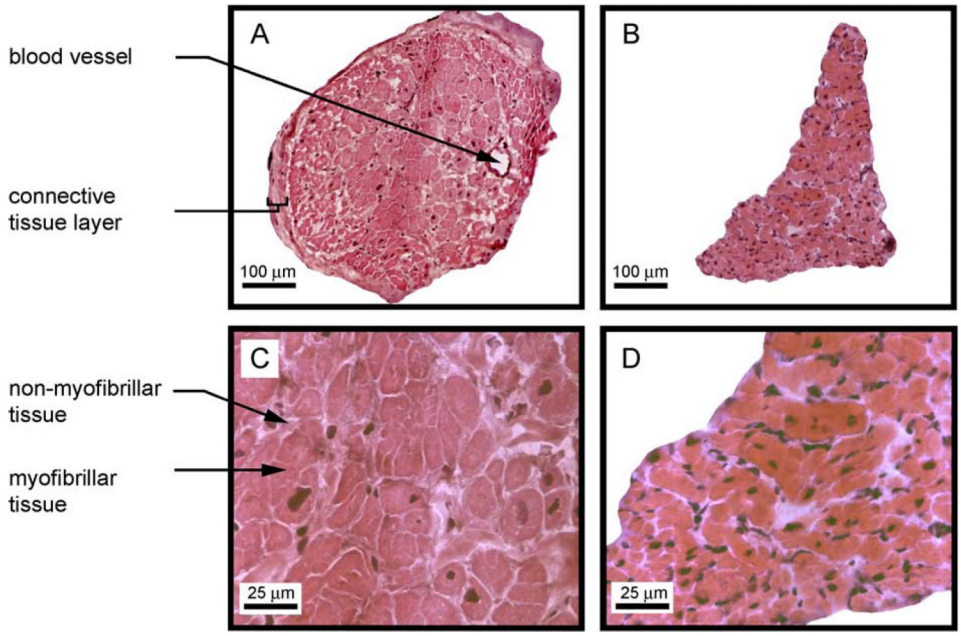

Figure 2.7. **Cross-sectional images of preparations.** Cross-sectional images of an atrial trabecula (**A, C**) and ventricular muscle strip (**B, D**) were made at 10x (**A, B**) and 40x (**C, D**) magnification. Atrial trabeculae have a surrounding layer of connective tissue and occasionally contain blood vessel(s). The atrial trabecula shown in panel A contains one blood vessel. Dark red structures correspond to myofibrillar tissue, while lighter parts correspond to the non-myofibrillar tissue.

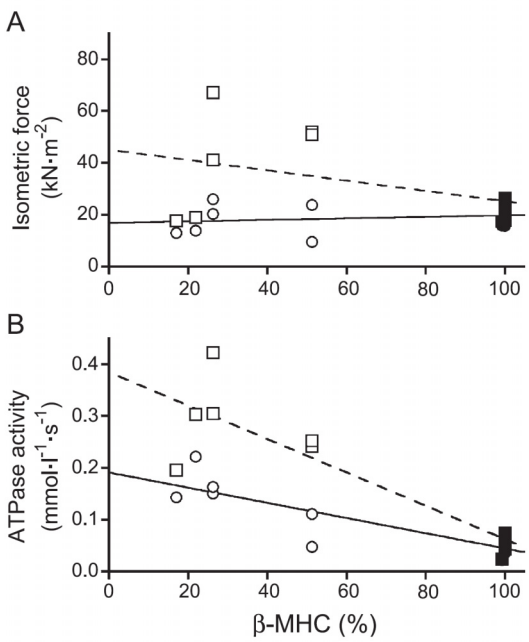

Figure 2.8. **Correction of data for the amount of myofibrillar tissue.** The correction of isometric force per cross-sectional area (**A**) and ATPase activity per volume (**B**) was performed in 6 atrial (open symbols) and 5 ventricular (closed symbols) preparations and the regression lines to the uncorrected (continuous line, circles) and corrected data (dotted line, squares) are shown. Isometric force did not depend on β-MHC composition for both uncorrected and corrected data. Regression line of uncorrected values: isometric force/cross-sectional area (in $kN \cdot m^{-2}$) = (16.8±3.5) + (0.03±0.05)× β-MHC (in %) (r^2=0.04, P=0.57). Regression line of corrected values: isometric force/cross-sectional area (in $kN \cdot m^{-2}$) = (45.1±10.3) - (0.2±0.1)× β-MHC (in %) (r^2=0.18, P=0.20). Correction enhanced the correlation between β-MHC composition and ATPase activity. Regression line of uncorrected values: ATPase activity/volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.19±0.02) – (0.0015±0.0003)× β-MHC (in %) (r^2=0.72, P<0.05). Regression line of corrected values: ATPase activity/volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.38±0.04) – (0.0032±0.0006)× β-MHC (in %) (r^2=0.79, P<0.05).

Discussion

This study shows that force development in human ventricular tissue is about 5 times more economical, though 9 times slower than in atrial myocardium and provides evidence that these differences are largely attributable to the difference in myosin heavy chain isoform composition. Significant linear relations were found between the MHC composition and ATP consumption and rate of force redevelopment. Extrapolation of these relations indicated that ATP consumption of the pure α-MHC was 5 times higher, whereas force redevelopment was considerably faster than observed for the β-MHC. It should be noted, however, that the extrapolated values have to be considered with caution because the scatter in the data is rather high. In addition, the linear relations used for the data analysis implicitly imply that myosin molecules act as independent force generators. Evidence suggests that this might not necessarily be the case.[59,68] However, even if interaction between myosin heads does occur the functional differences between atrial and ventricular tissue would remain. In fact, the concave relationships found[59,68] would imply that the difference between the properties of the pure isoforms would become even larger. Within atrial tissue, the correlation between MHC composition and rate of force redevelopment was significant (r^2=54, $P<0.05$), and although the correlation between MHC composition and ATP consumption for atrial samples did not reach statistical significance ($P=0.18$), its functional relation was rather similar to the relationship found for the combined set of atrial and ventricular data.

Methodological considerations

It should be noted that isometric force and ATP consumption were determined at 20°C, whereas K_{TR} values were determined in cardiac myocytes at 15°C. These experimental temperatures were chosen to facilitate comparison with previous studies in rat and human, from which it also became apparent that at higher temperatures sarcomere uniformity and stability of the cardiomyocytes during the measurements were not well preserved. We do not expect a difference in temperature dependency of functional properties between atrial and ventricular tissue, but caution should be exerted in extrapolation of the values found in this study to body temperature.

During activation inorganic phosphate (P_i) accumulates in the preparations due to the ATP hydrolysis. Since P_i accumulation is proportional to the ATPase activity and the

square of muscle diameter, this accumulation will be approximately 0.17 and 0.70 mM respectively in the ventricular and atrial preparations used in this study. From the previous studies in rat[69] and human cardiac myocytes[22,67] it followed that such low amounts of P_i depress force by less than 5%, while the effect on ATPase activity will be even smaller. Since, P_i accumulation is somewhat larger in atrial than in ventricular tissue, it slightly reduces the difference in force and consequently, in tension cost between atrial and ventricular tissue. However, overall the impact of P_i accumulation on functional properties is very minor and therefore does not affect the conclusions of this study.

Ca^{2+}-sensitivity

The Ca^{2+}-sensitivities of force and ATPase activity observed both in atrial and in ventricular tissue were very similar (Table 2.1). This indicates that the difference in tension cost between atrial and ventricular tissue is independent of the free Ca^{2+} concentration and implies that the energetic differences observed also hold at sub-maximal Ca^{2+} concentrations found *in vivo*.

Effect of MHC composition

During human heart failure a shift has been observed from the fast α-MHC isoform to the slow β-MHC isoform both in atria and ventricles.[21,34] The marked difference in economy observed in this study, clearly indicates that even a minor shift is associated with an improvement of the economy of contraction, albeit at the expense of speed of force development. It can be calculated from our experiments that a 7% decline in α-MHC composition, as was found in failing human ventricles by Miyata et al.,[34] will cause a 20% reduction in maximal ATPase activity. Recently, it has been shown that expression of a small amount of α-MHC (~12%) in rat cardiomyocytes significantly increases power output,[70] whereas no significant differences in isometric force or actin sliding velocity were observed between nonfailing and failing human myosin with a minor (~3%) difference in the expression of fast myosin (V1).[71] Our results in human tissue are in line with the observations in rat cardiomyocytes[70] and suggest that enhanced economy is paralleled by a reduction in maximum power output.

Effect of regulatory proteins and their phosphorylation status

Although the catalytic subdomain of ATPase activity and the actin binding region are located at the head portion of myosin, other contractile proteins, such as the myosin light chains and the troponin complex may exert a modulatory role in contraction.[23,40,52,56,72-74] Part of the variability in contractile properties within and between atrial and ventricular tissue may be explained by the expression of tissue-specific isoforms and/or by differences in phosphorylation status of contractile proteins. For instance, protein kinase C mediated phosphorylation of troponin has been shown to reduce both maximum tension and actomyosin Mg-ATPase activity by ~30% in mouse myocardium.[52,73,74] It should be noted that these effects are relatively small compared to the 3.3-fold difference in ATPase activity between atrial and ventricular tissue. Moreover, if troponin phosphorylation induces a parallel change in force and ATPase activity, it would not affect the differences in economy observed in the present study.

Speed versus economy

The correlation between ATPase activity and K_{TR} (Figure 2.4) resembles previous observations on stable maintenance heat rate and rate of force redevelopment in skeletal muscle fibers.[75] In a simple 2-state model for crossbridge interaction[28] ATPase activity is governed by the rate limiting step in the crossbridge cycle: the rate of crossbridge detachment (g) and $K_{TR} = f + g$, where f equals the rate of crossbridge attachment. The proportionality observed between ATPase activity and K_{TR} thus suggests that either K_{TR} is dominated by g ($f >> g$) or that f and g covary in proportion in different mixtures of fast and slow cardiac MHC isoforms (i.e. $f = c \cdot g$; where c is constant). In either case maximal tension, which is proportional to $f / (f + g)$, would be expected to be similar in atrial and ventricular preparations, as was found experimentally after correction for myofibrillar tissue density (Table 2.1).

Cardiac adaptation to mechanical overload is likely governed by the trade-off between speed and economy of contraction.[76,77] This principle might also explain why the relatively “expensive” fast MHC-isoform predominates in the atria: the contribution of atrial contraction to ventricular filling is rather small (as are the associated energy costs) but it might gain importance, and promote cardiac output, at high heart rate. Moreover, it is noteworthy that in states of reduced ventricular compliance such as ventricular hypertrophy

and heart failure the atrial contribution to ventricular filling increases by approximately 20%.[78]

Conclusion

Since our data show that the slow isoform is 5 times more economical than the fast isoform, the switch towards the slow β isoform clearly has considerable impact on performance in human atrial and ventricular myocardium.

Chapter 3

Myosin heavy chain composition and the economy of contraction in healthy and diseased human myocardium

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Abstract

Changes in myosin heavy chain (MHC) isoform expression and protein composition occur during cardiac disease and it has been suggested that even a minor shift in MHC composition may exert a considerable effect on myocardial energetics and performance. Here an overview is provided of the cellular basis of the energy utilization in cardiac tissue and novel data are presented concerning the economy of myocardial contraction in diseased atrial and ventricular human myocardium. ATP utilisation and force development were measured at various Ca^{2+} concentrations during isometric contraction in chemically skinned atrial trabeculae from patients in sinus rhythm (SR) or with chronic atrial fibrillation (AF) and in ventricular muscle strips from non-failing donor or end-stage failing hearts. Contractile protein composition was analysed by one-dimensional gel electrophoresis. Atrial fibrillation was accompanied by a significant shift from the fast α-MHC isoform to the slow β-MHC isoform, whereas both donor and failing ventricular tissue contained almost exclusively the β-MHC isoform. Simultaneous measurements of force and ATP utilisation indicated that economy of contraction is preserved in atrial fibrillation and in end-stage human heart failure.

Introduction

Human cardiac tissue contains 2 isoforms of the myosin heavy chain (MHC): fast (α-) and slow (β-) MHC, which share 93% of structural homology in the rat.[57] Functional properties of these two isoforms have been studied mainly in rodent tissue. For instance, it was found in rat and rabbit ventricular tissue that the α-MHC isoform exhibits a two to three times higher actin-activated ATPase activity[58] and actin filament sliding velocity[59] than the β-MHC isoform. Recently, in experiments on skinned human atrial and ventricular preparations we found that the β-MHC isoform is approximately 5 times more economical, but considerably slower than the α-MHC isoform.[79] Comparable observations were obtained in rodent models.[61,80,81]

In many forms of cardiac disease resulting in pressure or volume overload of the heart as well as with aging, a transition from α- to β-MHC takes place.[61,82] This could be advantageous, since less energy is required for force maintenance, but a shift towards more

β-MHC also reduces the speed of contraction and relaxation.[83,84] This compromises diastolic function of the ventricles at higher heart rates. Moreover, the contribution of the atrial contraction to filling of the ventricle may be reduced by alterations in the timing of the contractions. For instance, a recent study in transgenic mice showed that a shift is disadvantageous under severe cardiovascular stress.[85]

Interestingly, similar examples of muscle plasticity are found in skeletal muscle: aging, immobilization, bed-rest and microgravity are all associated with a shift in the MHC distribution from slow to fast isomyosins.[86-89] In addition, our group recently found in skeletal muscle fibres from patients with chronic heart failure evidence for a marked almost 2-fold reduction in force development and ATP utilisation in comparison with fibres of the same type from healthy age-matched control subjects.[90] This latter observation suggests that apart from a shift in MHC isoforms, also structural and posttranslational modifications may occur in skeletal muscle. Posttranslational modifications, such as altered phosphorylation, are prominent in diseased myocardium and influence contractile function.[40,63,91,92]

In the heart differences are observed in the MHC isoform composition of the atria and the ventricles, which are age and species dependent.[61,93-96] The general tendency is that with an increase in body size and age the expression of the slow β-MHC isoform becomes more pronounced. In addition, in human atrial tissue, an approximately one-to-one ratio is found between the fast and slow MHC isoforms, whereas in the ventricles the slow MHC isoform predominates. Evidence that an age-dependent shift may be operative in human ventricles has been obtained recently.[97] In addition, regional expression patterns in the regulatory myosin light chain are evident in diseased ventricular tissue,[98] which may also have functional implications.

The expression patterns also vary with cardiac hypertrophy (e.g.[99]). It has been shown in human ventricular tissue that the proportion of α-MHC mRNA of total MHC mRNA was ~30% in non-failing and was reduced to ~2% in end-stage failing hearts.[100,101] This group of investigators also showed that the efficiency of translational regulation is variable: in 12 non-failing hearts 35% of α-MHC mRNA was present, but only 7% α-MHC protein was expressed, similarly, in end-stage failing hearts, despite of the presence of a low amount of α-MHC mRNA, no α-MHC protein was detected.[34]

An important question is whether the relatively small changes in MHC composition in human tissue have functional relevance. From the difference in the maximum shortening velocity of the fast and slow isoforms and because of the usually observed increased steepness of the force-velocity relation in slow myosins, we hypothesized that power output, the mathematical product of force and velocity, might vary strongly with even small shifts in MHC composition. Evidence in favour of this was obtained in rat cardiac tissue.[70] Therefore, we investigated functional properties and contractile protein composition in both healthy and diseased human ventricular and atrial tissue. Muscle strips were isolated from ventricular donor and failing explanted hearts and trabeculae were excised from atrial biopsies obtained during bypass and valve surgery of patients in sinus rhythm and chronic atrial fibrillation. ATPase activity and isometric force development were measured simultaneously in these preparations at various Ca^{2+} concentrations.

Materials and methods

Biopsies

Atrial samples were obtained from right atrial appendages during coronary bypass and valve replacement surgery on patients with sinus rhythm (SR) (n=15; age 60±3 years) and normal left ventricular function and patients with chronic atrial fibrillation (AF) (n=12; age 69±3 years), who had moderate or bad left ventricular function. Duration of atrial fibrillation was ≥ 3 months.

Left ventricular biopsies were obtained during heart transplantation surgery from 6 non-failing donor hearts (age 41±5 years) and 6 explanted end-stage failing (New York Heart Association class IV) hearts (55±4 years). Heart failure resulted from ischemic (n=5) or dilated (n=1) cardiomyopathy. Biopsies were divided in two parts: one part was used for protein analysis, and the other part was used to isolate preparations for subsequent isometric force and ATP consumption measurements. Samples were obtained after informed consent and with approval of the local ethical committees. The investigation conformed with the principles outlined in the Declaration of Helsinki. Clinical characteristics of the patients are presented in Table 3.1.

Table 3.1. **Clinical characteristics of patients**

#	Gender	NYHA class	Operation/Disease	Medication
ATRIAL				
SR				
1	M	I/III	CAB	Asa, β, Ca^{2+} ant , nitr, s it
2	M	II	AVR	Asa, β, nitr, stat
3	F	/II	AVR	-
4	M	II	CAB	Asa, ACE, nitr, diur, st
5	F	V	CAB	Asa, ACE, β, Ca^{2+} ant, at
6	M	V	CAB	Asa, β, nitr, stat
7	M	I/III	CAB	Asa, β, Ca^{2+} ant, ATII a t, stat
8	M	I	CAB , I	ACE, Ac, β
9	M	I/III	CAB	Asa, β, nitr, stat
10	M	I	CAB	Asa, β, nitr, stat
11	M	V	CAB	Asa, β, Ca^{2+} ant, nitr, st t
12	M	I/III	CAB , I	Asa, β, stat
13	M	I	CAB	Asa, β, Ca^{2+} ant, nitr, st t
14	M	II	CAB /AVR	Asa, β, nitr
15	M	II	CAB	Asa, β, nitr, stat
AF				
1	M	II/IV	CAB , I	Ac, ACE, ATII ant,dig, tat
2	M	V	MVR	Ac, ACE, dig, diur
3	M	II	CAB	Ac, β, dig, diur
4	M	V	MVR	Ac, ACE, β, diur
5	F	II	MVR	Ac, ACE, β, dig, diur
6	M	II	CAB , I	Ac, ATII ant, β, diur
7	M	II	CAB /AVR/MVR	Ac, dig, diur, nitr
8	M	I	CAB /AVR	Ac, ACE
9	F	II	CAB /AVR, DM	Ac, ACE, Ad, dig, diur
10	M	I	MVR TVR	Ac, dig, diur
11	F	II	MVR TVR	Ac, ACE, β, diur
12	M	II	CAB	Asa, Ac, ACE, β, dig, r
VENTRICULAR				
D				
1	F	.	-	-
2	M	.	-	-
3	M	.	-	-
4	F	.	-	-
5	M	.	-	-
6	F	.	-	-
HF				
1	M	V	DCM	Ac, ACE, diur
2	M	V	ISCM	Ac, ACE, diur, dig, PI
3	M	V	ISCM	Ac, ACE, diur, nitr, PI
4	F	V	ISCM	Ca^{2+} ant
5	M	V	ISCM	Ac, ACE, diur, dig
6	F	V	ISCM	Ac, ACE, diur, PI

Abbreviations: SR: sinus rhythm, AF: atrial fibrillation, D: donor, HF: heart failing, M: male, F: female, AVR/MVR/TVR: atrial (mitral or tricuspid) valve replacement, CABG: coronary artery bypass grafting, DCM- dilated cardiomyopathy, ISCM- ischemic cardiomyopathy, DM: diabetes mellitus, I: old infarction. Medication: Ac: anticoagulants, ACE: ACE inhibitor, Ad: anti-diabetic, Asa: acetyl salicylic acid, ATII ant: ATII antagonist, β: beta blockers, Ca^{2+} ant: calcium antagonist, diur: diuretics, dig: digoxine, nitr: nitrates, PI : positive inotropic agent, stat: statine.

Protein analysis

The myosin heavy chain isoform composition was analysed by one-dimensional SDS polyacrylamide gel electrophoresis (1D SDS-PAGE).[40,61] The separating gel contained 12% total acrylamide (acrylamide to bis-acrylamide ratio 200:1; pH 9.3), while the stacking gel contained 3.5% total acrylamide (acrylamide to bis-acrylamide ratio 20:1; pH 6.8). Gels were stained with silver and analysed by laser densitometry. To check for linearity, different amounts of atrial and ventricular tissue (0.2-1.0 μg) were loaded and the density of the MHC bands was analysed. Based on these determinations 0.5 μg was chosen for sample application as it was found to be within the linear range.

Isolation of atrial trabeculae and ventricular strips

All preparations were isolated as described earlier.[79] Briefly, 21 SR and 23 AF atrial trabeculae were isolated in cold relaxing solution (pH 7.0; in mmol/l: free Mg^{2+} 1, KCl 145, EGTA 2, ATP 4, imidazole 10). Ventricular muscle strips (13 donor and 16 failing) were dissected parallel to the long axis of the muscle cell orientation. The mean dimensions (± S.E.M.) of atrial trabeculae amounted to 1.61±0.11 and 1.50±0.13 mm in length, 431±24 and 404±25 μm in width, and 380±22 and 373±25 μm in depth, for SR and AF group respectively. For ventricular donor and failing preparations mean dimensions were 2.36 ±0.17 and 2.17±0.23 mm in length, 472±41 and 398±33 μm in width, and 495±34 and 374±23 μm in depth, respectively. After isolation, all preparations were chemically permeabilised in relaxing solution with 1% Triton X-100 overnight (4°C). Next, the preparation was mounted between a force transducer and a fixed hook by means of aluminium T-clips.

Isometric force and ATP consumption measurements

The experimental procedures and equipment used were as described previously.[62,79] ATPase activity was measured by enzymatic coupling of ATP resynthesis to the oxidation of NADH, which could be quantified photometrically from the absorbance of near-UV light. Standard relaxing, preactivating and activating solutions were used.[64] The pCa ($-\log_{10}[Ca^{2+}]$) of the relaxing and maximally activating solution were, respectively, 9 and 4.5. Solutions with intermediate $[Ca^{2+}]$ were obtained by mixing of the activating and relaxing

solutions. Isometric force and ATPase activity were measured at 20±1°C. Maximum force and ATPase activity were determined when a steady-state force was reached. On average, force decline between the first and the last maximal control activation amounted to 18±1% and 20±2% for SR and AF atrial preparations, and to 10±2% and 8±3% for donor and failing ventricular preparations, respectively. The Ca^{2+}-activated ATPase activity was determined by subtraction of basal rate of ATP utilisation (measured in relaxing solution) from total rate of ATP utilisation measured in activating solution with various Ca^{2+} concentrations. The length of the preparations was adjusted on the basis of passive tension by stretching them to 1-2 kN/m^2, which corresponds to a sarcomere length of about 2.2 μm.[79] The cross-sectional area of the preparations was estimated assuming an elliptical shape.

Histochemical analysis

Histochemical analysis was performed as described previously.[79] At the end of the experiment preparations were embedded in relaxing solution containing 15% (w/v) gelatine and frozen in liquid nitrogen. Tissue sections (5 μm) were cut and subsequently stained with hematoxylin and eosin. Afterwards the sections were studied as described by des Tombe et al.[66] using a Leica DMRB microscope (Wetzlar, Germany). The myofibrillar area and interstitial space were determined relative to the cross-sectional area and expressed as percentages.

Data analysis

Force-pCa and ATPase-pCa relations were fit to a Hill equation:[67]

$$F(Ca^{2+})/F_0=[Ca^{2+}]^{nH}/(Ca_{50}{}^{nH}+[Ca^{2+}]^{nH}),$$

where F is steady state force (or ATPase activity). F_0 denotes the steady force (or ATPase activity) at saturating Ca^{2+} concentration, nH reflects the steepness of the relation, and Ca_{50} (or pCa_{50}) represents the midpoint of the relation.

All data were averaged per patient. Values are given as means ± S.E.M. of *n* patients. All statistical statements are based on two tailed Student's *t*-tests with a 0.05 level of significance. Linear regression analysis was performed using Prism 4.0 (GraphPad Software Inc).

Results

Contractile protein composition

MHC isoform composition was analysed by one-dimensional SDS-PAGE (Figure 3.1A, B). Densitometric analysis of the gels revealed that atrial tissue of patients in the sinus rhythm group (SR) consisted of a mixture of α- and β-MHC and that in the atrial fibrillation group (AF) a shift towards more β-MHC occurred (Table 3.2, Figure 3.1B). The mean values of β-MHC isoform content were significantly different between the SR and AF groups, but the ranges of MHC isoform distribution were rather similar (from 6.9 to 51.3% of total MHC content in SR and from 12.3 to 62.0% in AF). Ventricular samples predominantly contained the β-MHC isoform: only in two donor and one failing heart sample was the α-MHC isoform detected.

Isometric force and ATPase activity

Maximal isometric tension (i.e. force divided by cross-sectional area) did not significantly differ, neither between SR and AF (13.9±1.0 versus 11.0±1.5 kN/m^2) nor between donor and failing (18.6±2.3 versus 14.6±4.3 kN/m^2) myocardium. Also no difference was found in maximal ATPase activity between healthy and diseased myocardium both in atrial and ventricular tissue (Table 3.2, Figure 3.2A, B). However, on average maximal ATP consumption was significantly higher in atrial than in ventricular tissue.

The average force-pCa and ATPase activity-pCa relationships obtained in atrial and ventricular preparations are shown in Figure 3.3. It can be noted that in atrial tissue Ca^{2+}-responsiveness of force was not significantly different between SR and AF groups, whereas Ca^{2+}-responsiveness of ATP consumption was significantly higher in AF compared to SR tissue (Table 3.2; Figure 3.3A). In ventricular tissue Ca^{2+}-sensitivity of both force and ATPase activity was increased in failing hearts compared to donor myocardium (Table 3.2, Figure 3.3B; $P<0.05$). The steepness of the force-pCa and ATP-pCa curves, nH, did not differ significantly between SR and AF atrial and donor and failing ventricular myocardium (Table 3.2).

Tension cost, a measure of muscle economy, which gives information about the rate of ATP splitting necessary for maintenance of isometric force, was calculated by dividing the ATPase activity per volume by force per cross-sectional area. The mean tension cost was significantly different between atrial and ventricular cardiac tissue, but did

not differ significantly neither between SR and AF atrial nor between donor and failing ventricular myocardium (Table 3.2, Figure 3.2C).

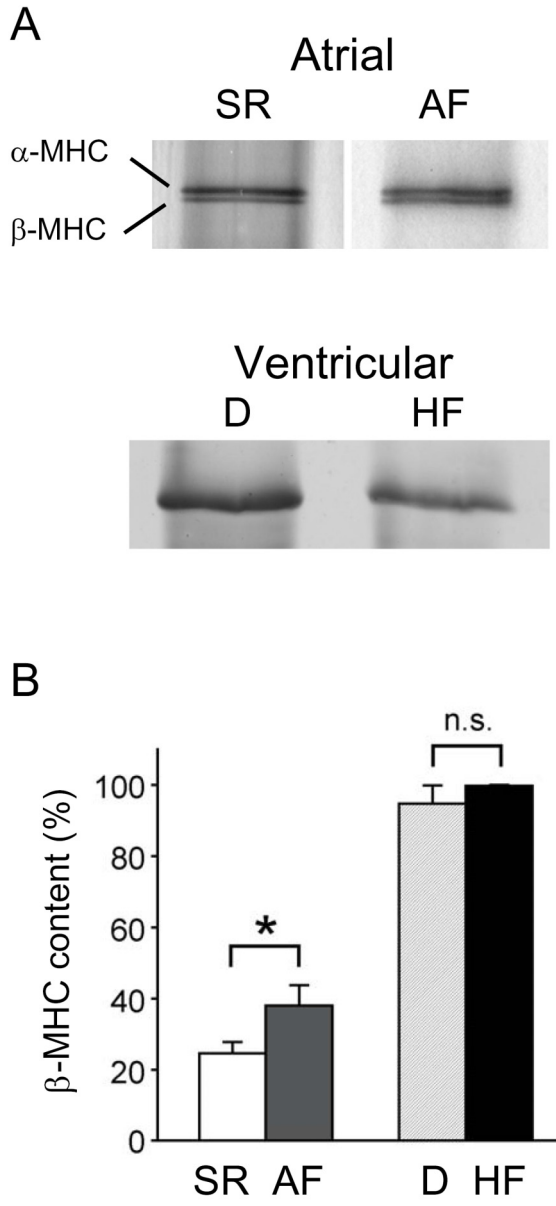

Figure 3.1. **MHC isoform composition. A.** Detail of the myosin heavy chain area of SDS-polyacrylamide gels from atrial tissue of a patient in sinus rhythm (SR) and a patient with chronic atrial fibrillation (AF) and from ventricular tissue of a donor heart (D) and an end-stage failing heart (HF). **B.** Mean values of β-MHC content in the different groups, determined by laser densitometry.

Table 3.2. **Mean parameters in atrial and ventricular tissue**

Parameter	Atrial		Ventricular	
	SR (n=15)	AF (n=12)	D (n=6)	HF (n=6)
β-myosin heavy chain content (% of total MHC)	24.6±3.2	38.1±5.7*	94.7±5.2	99.8±0.17
Ca^{2+}-activated ATPase activity per preparation volume ($mmol \cdot l^{-1} \cdot s^{-1}$)	0.15±0.01	0.13±0.02	0.05±0.01	0.05±0.02
Basal ATPase activity at pCa 9 (% of total)	10.9±0.8	13.9±1.4	11.4±1.4	14.3±2.1
Maximal tension ($kN \cdot m^{-2}$)	13.9±1.0	11.0±1.5	18.6±2.3	14.6±4.3
pCa_{50} force	5.66±0.02	5.72±0.04	5.67±0.04	5.86±0.02#
nH force	3.09±0.19	2.81±0.17	2.29±0.29	2.75±0.26
pCa_{50} ATPase activity	5.69±0.03	5.80±0.04*	5.68±0.04	5.90±0.03#
nH ATPase activity	2.52±0.11	2.55±0.25	2.36±0.35	2.26±0.29
Tension cost ($mmol \cdot kN^{-1} \cdot m^{-1} \cdot s^{-1}$)	11.4±1.3	12.0±1.4	2.4±0.3	3.7±0.9
Myofibrillar tissue (%)	55±6	44±7	89±2	75±9
Interstitial space (%)	20±4	44±7*	11±2	19±2
Maximal tension corrected for myofibrillar tissue ($kN \cdot m^{-2}$)	28.4±6.3	30.7±3.7	21.1±2.1	32.5±5.6
Ca^{2+}-activated ATPase activity per preparation volume corrected for myofibrillar tissue ($mmol \cdot l^{-1} \cdot s^{-1}$)	0.23±0.03	0.34±0.04	0.05±0.01	0.12±0.05

Abbreviations: pCa = -log [Ca^{2+}]. pCa_{50}, Ca^{2+} concentration at which half of the maximal isometric force or ATPase activity is obtained; nH, steepness of pCa-force and pCa-ATPase activity relationships. Values are given as mean ± S.E.M. of *n* patients. Myofibrillar tissue and interstitial space were determined for 9 and 15 preparations of SR and AF tissue, and 5 and 3 muscle strips of D and HF tissue, respectively. *significant at P<0.05, atrial SR versus AF; # ventricular D versus HF.

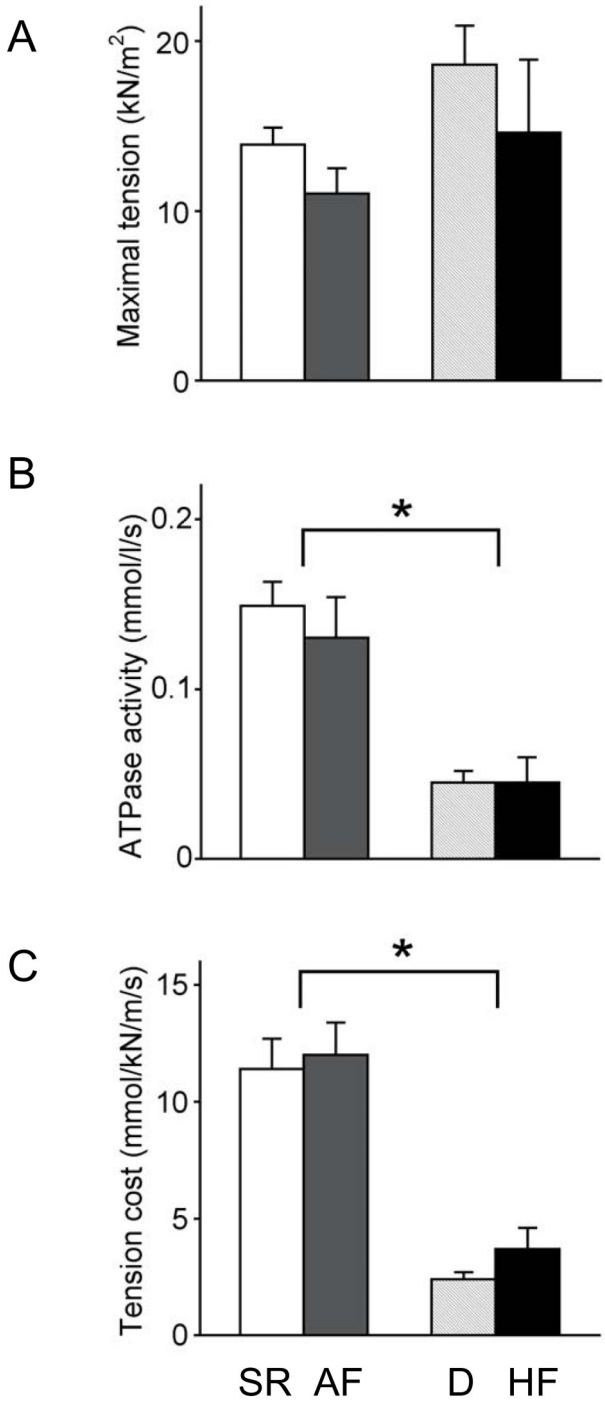

Figure 3.2. **Maximum isometric force, ATPase activity and tension cost.** Maximum isometric force per cross-sectional area (**A**), ATPase activity per preparation volume (**B**) and tension cost (**C**) in atrial (SR, AF) and ventricular (D, HF) tissue. The differences in ATPase activity and tension cost were statistically significant (ANOVA; $P<0.05$) between atrial and ventricular tissue (*). No statistically significant differences were found in maximal tension between atrial and ventricular tissue, nor in any of the parameters *within* groups, in SR vs. AF or D vs. HF.

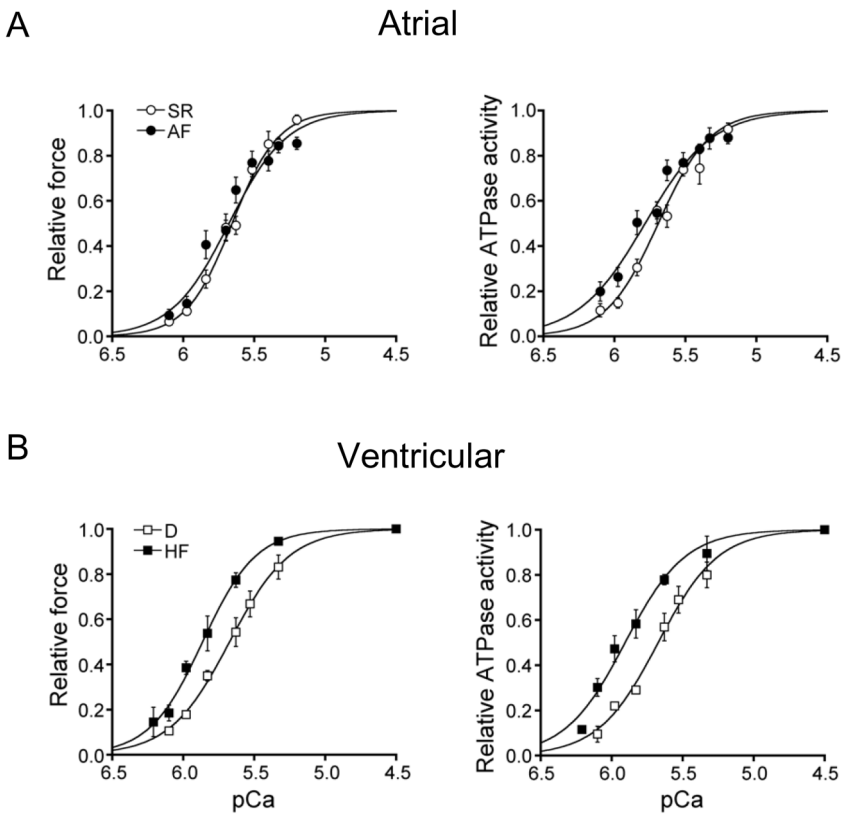

Figure 3.3. **Ca^{2+}-sensitivity of isometric force and ATPase activity: atrial (A), ventricular (B) tissue.** Isometric force and the rate of ATP utilisation at submaximally activating Ca^{2+} concentrations are normalized to the control values found at saturating Ca^{2+} concentration (pCa 4.5). Mean values± S.E.M. are shown. The Hill curves defined in Materials and Methods were fitted to the mean data using their S.E.M.-s as a weight factor.

Morphological structure of preparations

Histochemical analysis was performed on 24 atrial preparations (9 SR, 15 AF trabeculae) and 8 ventricular (5 donor, 3 failing muscle strips) after determination of force and ATPase activity. It was found that the average amount of myofibrillar tissue was not significantly different between SR and AF atrial (55±6 versus 44±7%) and donor and failing ventricular preparations (89±2 versus 75±9%, Table 3.2). However, the percentage of interstitial space in SR preparations (20±4%) was significantly lower than in AF (44±7%). The interstitial

space in ventricular strips from donors was also lower than in failing strips, but this difference did not reach statistical significance (P=0.06; Table 3.2).

The variable amount of myofibrillar tissue within atrial and ventricular preparations may alter force characteristics and energy consumption. To investigate this point, maximal tension and ATP consumption were corrected for the proportion of myofibrillar tissue. This did not result in a difference in maximal tension, but enlarged the difference in ATPase activity between atrial and ventricular tissue (Table 3.2).

Correlation between functional properties and MHC composition

A significant negative correlation was found between the rate of ATP consumption and tension cost of atrial and ventricular preparations and their MHC composition (Figure 3.4A, B) (r^2=0.50 and r^2=0.53 respectively, P<0.05). Maximal tension did not depend on MHC composition (figure not shown).

After correction for myofibril density, the correlation between ATPase activity and β-MHC composition in a subset of the preparations used for histochemical analysis (*n*=32) slightly improved, as was reflected by an increase in the r^2 value from 0.28 for the uncorrected data to 0.30 for the corrected data (Figure 3.4C).

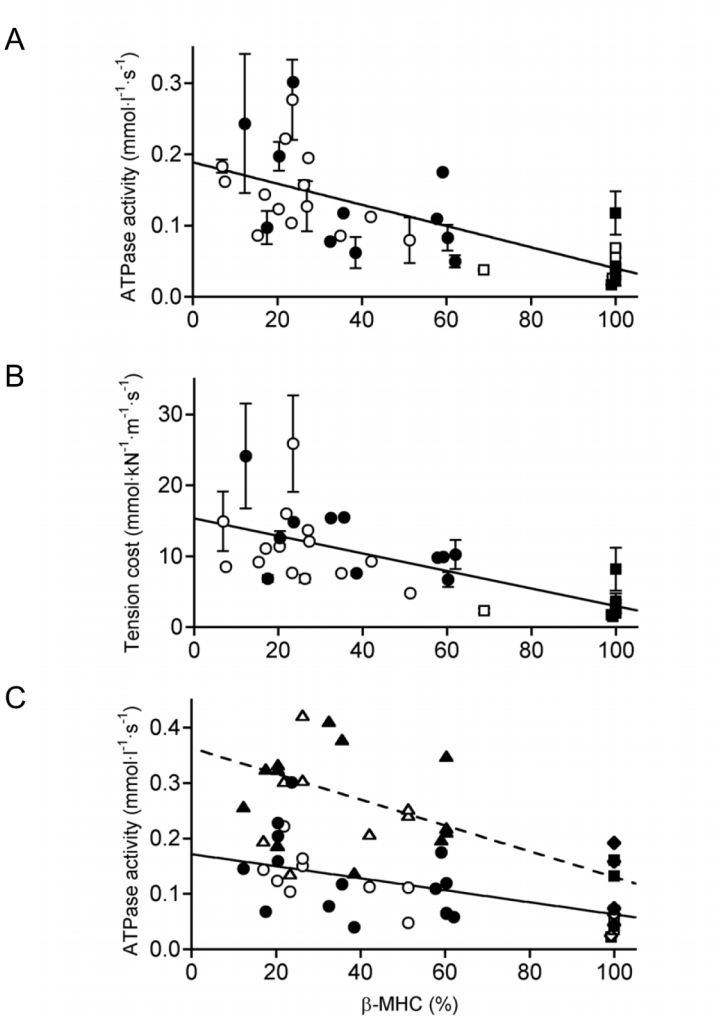

Figure 3.4. **Relation between β-MHC expression and ATPase activity or tension cost. A.** ATPase activity averaged per patient. A significant negative correlation was found between β-MHC fraction (% of total MHC) and mean ATPase activity (SR *n*=14, AF *n*=11, D *n*=6, HF *n*=6). Regression line: ATPase activity/volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.19±0.02) – (0.0015±0.0003)× β-MHC (in %) (r^2=0.50, P<0.05). **B.** Tension cost averaged per patient. A significant negative correlation was found between β-MHC fraction (% of total MHC) and mean tension cost (SR *n*=14, AF *n*=11, D *n*=6, HF *n*=6). Regression line: tension cost (in $mmol \cdot kN^{-1} \cdot m^{-1} \cdot s^{-1}$) = (15.4±1.2) – (0.12±0.02)× β-MHC (in %) (r^2=0.53, P<0.05). **C.** ATPase activity averaged per total preparation volume and per myofibrillar volume. Significant negative correlations were found between β-MHC (%) and mean ATPase activity (SR *n*=9, AF *n*=15, D *n*=5, HF *n*=3). Regression lines: ATPase activity/total volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.17±0.02) – (0.0011±0.0003)× β-MHC (in %) (r^2=0.28, P<0.05); ATPase activity/myofibrillar volume (in $mmol \cdot l^{-1} \cdot s^{-1}$) = (0.36±0.04) – (0.0023±0.0006)× β-MHC (in %) (r^2=0.30, P<0.05). Meaning of symbols: open circles, SR; filled circles, AF; open squares, donor; filled squares, HF; open triangles: SR after correction for non-myofibrillar volume; filled triangles: AF after correction; open diamonds: donor after correction; open triangles: HF after correction.

Discussion

Summary of the findings

In atrial tissue a significant shift from the α- to the β-MHC isoform was observed in atrial fibrillation, whereas in ventricular failing myocardium no systematic changes were found as compared to donor tissue. Overall a significant and strong negative correlation was found between β-MHC content and ATPase activity. This is consistent with the notion that the MHC isoform shift in diseased states has a considerable impact on muscle energetics. However, the functional properties of the myosin heavy chain isoforms were not affected in health and disease.

Structural and functional differences in healthy atrial and ventricular human myocardium

To assess the mechanical and energetic properties of atrial and ventricular tissue, both their contractile protein composition and myofibrillar density need to be taken into account. In healthy human atrial tissue an approximately equal distribution is found between the fast and slow MHC isoform, whereas in the ventricles the slow MHC isoform predominates. Our data obtained in permeabilised preparations enable us to assess the implications of structural differences in myofibrillar density on force production and the rate of ATP utilisation. These parameters are equally influenced by myofibrillar density. Hence tension cost, a measure of economy, does not dependent on density. However, to extrapolate the findings to cardiac performance *in vivo*, effects arising from permeabilisation (i.e. washout of mitochondria and possibly myofibrillar swelling[102,103]) and alterations in geometry of the heart during disease need to be taken into account.

After correction for non-myofibrillar space, force in atrial tissue was 1.3-fold larger than in ventricular tissue. In addition, the economy of force maintenance in atrial tissue was approximately 5-fold larger than in ventricular tissue. In these respects, atrial tissue resembles fast and ventricular tissue resembles slow skeletal muscle. In terms of crossbridge kinetics, it can be concluded that the rate of crossbridge formation (*f*) and of crossbridge dissociation (*g*) co-vary roughly in proportion, but that in atrial tissue *f* is increased more that *g* as compared to ventricular tissue. Since atrial tissue contains more α- than β-MHC, this also applies to the kinetics of the pure MHC isoforms.

MHC shift in atrial fibrillation

Atrial fibrillation is the most prevalent sustained arrhythmia affecting 2.2 million adults only in the United States every year.[104] Although contractile remodelling of atria in atrial fibrillation has been described already long time ago,[105] the molecular mechanisms underlying contractile alterations are still poorly resolved.

In our study we have also found, on average, a 13.5% increase in β-MHC content in AF cardiac tissue. It should also be noted that β-MHC isoform content in AF tissue varied considerably (range from 12.3 to 62.0%), which can explain why no significant differences were observed in the mean values for ATPase activity and tension cost between SR and AF groups.

It is known that rat cardiac myocytes with predominant β-MHC develop lower power output and velocity of contraction than those with mostly α-MHC.[70] The influence of MHC alterations in fibrillating atria is, presumably, rather small, since the atrial contribution under these conditions is minor. However, the impact of an isoform shift will become apparent upon cardioversion. Then a shift to β-MHC may be detrimental since it may contribute to blood stasis and consequently promote clot formation, embolism and stroke in AF patients.[106]

Ca^{2+}-sensitivity of ATP consumption was significantly higher in AF than in SR patients, while Ca^{2+}-sensitivity of force did not differ. This implies that tension cost in AF patients decreases with an increase in Ca^{2+} concentration. There are two possible explanations for this observation. The first possibility would be the presence of an additional ATPase in AF, with a lower EC_{50} than the actomyosin ATPase, which is not or incompletely eliminated by the Triton treatment. The second possibility would be that in AF patients the actomyosin ATPase is more sensitive to Ca^{2+} than the force development. All methodological factors, which artefactually could give rise to a discrepancy between the Ca^{2+}-sensitivity of ATPase and force (ADP and/or inorganic phosphate accumulation, internal shortening) have the opposite effect. It should be noted that an increased Ca^{2+}-sensitivity of ATPase activity would be detrimental, since more ATP would be required to maintain force.

Noteworthy is the observation that atrial tissue from AF patients had a significantly higher amount of interstitial space if compared to patients in SR. However, one should be careful relating this solely to atrial fibrillation. It was shown that valve

disease itself could cause fibrosis.[107] Since the AF group consisted mostly of patients suffering from valve insufficiency in contrast to the SR group with mostly coronary bypass patients, both factors (valve disease and AF) can be responsible for the observed fibrosis.

MHC isoform shift in ventricular failing hearts

In human ventricles the β-MHC isoform is predominant and amounts to 90-99% of total MHC composition. Thus, only a minor shift toward more β-MHC in failing ventricles is possible.[34] Based on the steep relation between ATP consumption and MHC composition even a small shift in MHC composition could alter myocardial pump function and energetics.[79] However, in the present study no differences were found in MHC composition and functional properties at maximal activation between healthy and failing human ventricular tissue indicating that an MHC shift is not a prominent feature under pathological conditions in ventricles as it is in the atria.

In ventricular failing myocardium both force and ATP utilization were more sensitive to Ca^{2+} than in donor ventricular tissue. A similar observation was made in single cardiomyocytes isolated from donor and failing human hearts.[39,40] The increased myofilament Ca^{2+}-responsiveness in failing ventricular myocardium results most likely from post-translational modification of proteins. Adrenergic stimulation of the heart results in phosphorylation of troponin I and a reduction in Ca^{2+}-sensitivity. In human heart failure, overstimulation of the β-adrenergic receptors leads to desensitisation of the β-adrenergic pathway. As a result troponin I phosphorylation is decreased in human failing ventricular tissue, which may explain the increased Ca^{2+}-responsiveness.[40]

MHC isoform shift in diseased myocardium, adaptive or maladaptive?

The question, which remains to be answered, is whether the changes in MHC composition are beneficial for patients or on the contrary, aggravate the disease. Based on the correlation found in our study we can conclude that the mean shift toward β-MHC observed in AF would result in a decrease in ATP utilisation by 13%. As has been noted previously[79] it seems beneficial that ventricles, which deliver most of the cardiac work, contain predominantly β-MHC isoform, which is slow, but more economical, i.e. require less

energy during isovolumic contractions. Similarly, the increase in β-MHC content in atria has a compensatory effect on heart function (schematically illustrated in Figure 3.5).

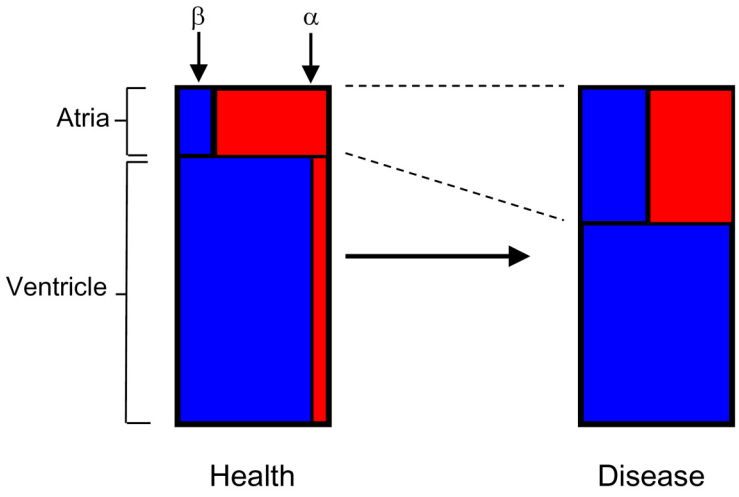

Figure 3.5. **Changes in MHC composition in atria and ventricles during disease.** In healthy myocardium the contribution of the atria to the ventricular filling is relatively small (around 20%). In diseased myocardium the role of the atrial contraction to the myocardial pump function increases and a shift in MHC isoform composition occurs in both atria and ventricles.

At rest the atria, which contain a mixture of both MHC isoforms, play a relatively small role in ventricular filling and cardiac pump function, but their contribution to ventricular filling increases under stressful conditions or during physical exercise, where the speed of contraction becomes more important. Similarly, during heart failure (but not atrial fibrillation) the relative contribution of atria to cardiac pump function increases. Under critical pathological conditions saving of energy in order to preserve cardiac function at rest will be more important than maintaining heart function during exercise and cardiovascular stress.

In a recent *in vivo* study in transgenic mice with almost complete replacement of the normally predominant α-MHC isoform with β-MHC[85] cardiac contractile function was reduced. However, animals were healthy, had a normal life span, and had no cardiac pathology suggesting that such an MHC isoform shift does not induce disease on its own, but appears to be disadvantageous only under severe cardiovascular stress.

Conclusion

The shift towards the slow and more energetic β-MHC isoform occurs both in human diseased atrial and ventricular myocardium, but this shift is considerably smaller in ventricles than in atria. The shift in MHC composition towards the β-MHC isoform in atrial tissue may be beneficial under pathological conditions, despite a reduction in speed of contraction, since less energy is required to maintain pump function at rest.

Chapter 4

Exchange of C-terminal truncated troponin I into human cardiac muscle increases Ca^{2+}-responsiveness, but does not affect maximum force

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Submitted combined with Chapter 5.

Abstract

The specific and selective proteolysis of cardiac troponin I (cTnI) has been proposed to play a key role in human ischemic myocardial disease, including stunning. In this study the functional implications of cTnI proteolysis were investigated in human cardiac tissue. The predominant human cTnI degradation product ($cTnI_{1-192}$) and full length cTnI were expressed in *E. coli*, purified, reconstituted with the other cardiac troponin subunits and subsequently exchanged into human cardiac myofibrils and permeabilised cardiomyocytes, isolated from healthy donor hearts. Maximal isometric force and kinetic parameters were measured in myofibrils, using rapid solution switching. To determine the effect of $cTnI_{1-192}$ on Ca^{2+}-responsiveness of the contractile apparatus, force development was measured at various calcium concentrations in single cardiomyocytes. Protein analysis by 1D-SDS gel electrophoresis, Western Immunoblotting and 3D-imaging revealed that approximately 50% of endogenous cTnI was homogeneously replaced by $cTnI_{1-192}$ in both myofibrils and cardiomyocytes. Maximal tension was not affected, while the rates of force activation and redevelopment as well as relaxation kinetics were slowed down. Ca^{2+}-sensitivity of the contractile apparatus was increased in preparations containing $cTnI_{1-192}$ complex (pCa_{50}: 5.73±0.03 versus 5.52±0.03 for $cTnI_{1-192}$ and full-length cTnI exchanged cardiomyocytes, respectively). These results indicate that degradation of cTnI in human myocardium will mainly impair diastolic function.

Introduction

Myocardial ischemic disease is manifested in a mild form of reversible contractile dysfunction (stunning) and a devastating, irreversible form (necrosis). The latter may only be prevented by timely restoration of coronary perfusion but even then, reversible contractile dysfunction is frequently apparent. The specific and selective proteolysis of cardiac troponin I (cTnI) has been proposed to play a key role in human myocardial ischemia/reperfusion injury, including stunning.[38,45,46] cTnI is part of the cardiac troponin (cTn) complex, that in concert with tropomyosin (TM), regulates muscle contraction in response to a rise in intracellular Ca^{2+}. The troponin complex consists of 3 subunits: troponin C (cTnC), the Ca^{2+} binding protein; cTnI, which inhibits the actin-myosin

interaction and troponin T (cTnT), which transduces the Ca^{2+} binding signal to TM.[19] A recent study on crystal structures of human cTn by Takeda et al.[25] indicated that upon Ca^{2+} binding to the regulatory site of cTnC, the C-terminal portion of cTnI moves away from the actin filament, thereby altering the orientation and/or flexibility of cTn and TM relative to the actin filament. Hence, cTnI plays a pivotal role in transducing the Ca^{2+}-binding signal to activation of cardiac myofilaments and truncation of the cTnI C-terminus, as observed in human ischemic cardiac disease, might alter the Ca^{2+} induced force development of the myofilaments as well as the inhibition of force development at low Ca^{2+} concentrations.

In rodents, McDonough et al.[108] showed that with moderate ischemia/reperfusion cTnI is cleaved at its C-terminus resulting in a degraded subunit $cTnI_{1-193}$. Ischemia-induced cTnI degradation has been attributed to activation of the Ca^{2+}-dependent proteolytic enzyme calpain-1 by the Ca^{2+} overload during reperfusion.[109] Increased preload in the absence of ischemia has also been shown to initiate cTnI degradation.[110] In a post-infarct pig model, minor degradation of cTnI (<4%) was observed in the viable remodelled left ventricle.[111] The maximal force generating capacity of the myofilaments in these experiments was reduced compared to myocardium of sham-operated animals. In advanced human heart failure serum cTnI elevations have been reported.[112] In addition, cTnI degradation has been found in human cardiac tissue from coronary artery disease patients with different degrees of heart failure and a history of myocardial infarction. [38,45,46] The primary cTnI degradation product found in these hearts was identified as $cTnI_{1-192}$ (similar to $cTnI_{1-193}$ in rodent myocardium[108,109,113]). Hence, in humans C-terminal truncation of cTnI may also underlie impaired cardiac function in ischemia-induced cardiomyopathy. However, the direct effects of cTnI proteolysis on contractile function of human cardiac myofilaments have not been investigated in detail.

In this study protein engineering was combined with functional measurements to assess the direct effects of degradation of the cTnI C-terminus in human myocardium. Force measurements were performed in human cardiac myofibrils and cardiomyocytes, in which endogenous troponin complexes were partly replaced by complexes containing either human full-length cTnI or truncated $cTnI_{1-192}$. The advantage of this technique is that it allows to investigate the direct effect of cTnI degradation on contractility of human cardiac preparations without degradation of other contractile proteins (as e.g. follows calpain-1

treatment[48,114]) or other compensatory protein changes, which may develop in transgenic animals.

Force development at maximal and submaximal [Ca^{2+}] and kinetic parameters of force activation and relaxation in preparations containing $cTnI_{1-192}$ were compared to those containing full-length cTnI. Surprisingly, in the presence of $cTnI_{1-192}$ maximal isometric force was preserved, Ca^{2+}-sensitivity was increased and the kinetics of activation and relaxation of human cardiac preparations were slowed. These data were unexpected since previous studies showed a loss in maximum force and an acceleration of the actomyosin ATPase kinetics.[113,115,116] Our data indicate that C-terminal truncation of cTnI mainly impairs diastolic properties of human myocardium.

Materials and Methods

Preparation of recombinant troponin complex

The human cardiac full-length cTnI and truncated cTnI ($cTnI_{1-192}$) peptides were prepared using the standard F-moc synthesis. All human troponin subunits (human cTnI and $cTnI_{1-192}$, cTnT and cTnC) were expressed separately in *E. coli* and purified using ion-exchange chromatography. The purified troponin subunits were reconstituted into a full troponin complex by mixing the subunits in a 1:1:1 molar ratio. After 2 hours incubation at room temperature the concentration of urea (6M) and KCl (1M) were reduced by step-wise dialysis[117]. Purity and stoichiometry of both reconstituted complexes, containing either full-length cTnI (cTn^{FL}) or the primary cTnI degradation product (cTn^{DEG}), was verified by one-dimensional gel electrophoresis (described below). Protein complexes were stored at -80°C prior to the experiments.

Isolation of human myofibrils and cardiomyocytes

Left ventricular tissue was obtained from healthy donor hearts (n=2), frozen in liquid nitrogen and stored at -80°C. Samples were obtained after informed consent and with approval of the local ethical committees. The investigation conformed with the principles outlined in the Declaration of Helsinki.[118]

Single myofibrils or bundles of two to three myofibrils were prepared from these biopsies, as described previously.[119] Shortly, small pieces of frozen ventricular tissue (70-

100 mg) were defrosted in rigor solution (10 mM Tris, 132 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, 10 mM NaN_3, pH 7.1), dissected in a few thin muscle strips (diameter 0.5-1.0 mm) and permeabilised in rigor solution containing 1% Triton X-100 for 2 hours on ice. To remove Triton, myofibrils were washed in rigor solution 3 times. Thereafter muscle strips were homogenized with a blender for 10 s at 4 °C to produce a suspension of human cardiac myofibrils. Exchange of troponin complexes was started immediately after myofibril isolation. All solutions to which the myofibrils were exposed contained a protease inhibitor cocktail (0.2 mM PMSF, 0.01 mM leupeptin, 0.01 mM E-64 and 0.005 mM pepstatin, Sigma).

Single cardiac myocytes were mechanically isolated using the protocol described previously[60] except that the tissue was handled in cold rigor solution rather than in relaxing solution. The cells were permeabilised in rigor solution supplemented with 0.5% Triton X-100 for 5 min on ice, subsequently washed and used for exchange.

Exchange of human cardiac cTn complex

Exchange of human cardiac troponin complex into human myofibrils and myocytes was performed according the method of Brenner et al.[120] Myofibrils and cardiomyocytes were incubated overnight at 4 °C in an exchange solution containing 0.5 mg/ml (*ca* 6 μM) of human troponin complex (either cTn^{FL} or cTn^{DEG}). Composition of the exchange solution was in mM: imidazole 10, NaCl 170, $MgCl_2$ 5, $CaCl_2$ 3, EGTA 2.5, DTT 0.5, NaN_3 0.5, pH 6.9 and the protease inhibitor cocktail described above. After exchange the myofibril and cardiomyocyte suspensions were washed twice by centrifugation (at 4000g for 10 min at 10°C and 900g for 1 min at 4°C, for myofibrils and cardiomyocytes, respectively) to replace the exchange solution with rigor solution. Half of the suspension was used for mechanical experiments, and the remaining half was used for analysis of the amount of cTn exchange by one-dimensional gel electrophoresis.

Force measurements in exchanged human myofibrils and cardiomyocytes

The set-up used for rapid solution changes and to record force in single myofibrils was as described previously.[28,121] Briefly, myofibrils were mounted horizontally between a calibrated cantilever force probe and a motor. Sarcomere length and myofibril diameter were measured from video images (magnification 1800X, phase-contrast optics). The initial

length of attached myofibrils between the attachments (mean ± S.E.M.), respectively, amounted to 2.20±0.04 μm (*n*=17) in the cTn^{FL} exchanged myofibrils and to 2.19±0.04 μm (*n*=25) in cTn^{DEG} exchanged myofibrils.

Myofibrils were activated and relaxed by rapid (10 ms) switching between two continuous streams of relaxing (pCa 9; pCa = $-\log_{10}[Ca^{2+}]$) and activating (pCa 3.5) solutions flowing by gravity from a double-barrelled glass pipette placed at right angles to, and within 1 mm of, the preparation. A release–restretch protocol was used to measure the rate of force redevelopment (K_{TR}).[28] The rate of rise of force following the step decrease in pCa by fast solution switching (K_{ACT}) was estimated from the time required to reach 50% of the maximum isometric force.

Isometric force in single human cardiomyocytes was measured at different $[Ca^{2+}]$ (pCa ranging from 4.5 to 6.0) and at a sarcomere length of 2.2 μm as described previously.[60] Maximal activation at pCa 4.5 was used to calculate maximal calcium-activated isometric tension (i.e. F_{max}/cross-sectional area). The cross-sectional area of the preparations was calculated assuming an elliptical shape, i.e. cross-sectional area = (width × depth × π) / 4. Accurate determination of myocyte depth was done by projecting the myocyte via a small mirror onto the objective of the inverted microscope.[122] Mean dimensions (± S.E.M.) of the cTn^{FL} (*n*=14) and cTn^{DEG} (*n*=14) exchanged cardiomyocytes amounted to 48.5±2.6 and 58.3±5.1 μm in length, 18.2±1.6 and 16.7±1.5 μm in width, and 19.7±2.1 and 17.0±1.6 μm in depth, respectively. After maximal activation 4 to 5 measurements were carried out at submaximal $[Ca^{2+}]$ (pCa>4.5) followed by a maximal activation. Force values obtained in solutions with submaximal $[Ca^{2+}]$ were normalized to the interpolated maximal force values (pCa 4.5). On average force decline between the first and the last maximal control activation amounted to 10±3% and 14±1% for cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes, respectively. Experiments where force decline exceeded 25% were discarded. Passive force was determined as indicated in Figure 4.5. All mechanical experiments were performed at 15°C.

Protein analysis

The amount of cTnI exchange was analysed in cardiac samples in which endogenous cTnI was replaced by $cTnI_{1\text{-}192}$. It has been shown previously for rabbit psoas myofibrils that the

amount of human full length cTnI exchange was the same as for $cTnI_{1-192}$.[123] Hence, in the present study evaluation of both cTn^{FL} and cTn^{DEG} exchange into human cardiac preparations was based on the amount of $cTnI_{1-192}$ exchange. This analysis was performed using Coomassie stained one-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE) and was based on the reduction in the ratio between endogenous cTnI and α-actinin in cTn^{DEG} preparations compared to cardiac samples, which were incubated in exchange solution without exogenous cTnI. The α-actinin band was chosen as a measure of myofibrillar protein loading because its intensity was within the linear range and not affected by the exchange procedure.

The separating gel of 1D SDS-PAGE used for the extent of cTn^{DEG} exchange determination contained 15% total acrylamide (acrylamide to bis-acrylamide ratio 200:1; pH 9.3), while the stacking gel contained 3.5% total acrylamide (acrylamide to bis-acrylamide ratio 20:1; pH 6.8). Cardiomyocytes and myofibrils were dissolved in standard Laemmli sample buffer.[124] Gels were stained with 0.1% Coomassie solution and analysed by laser densitometry (LKB Produkter AB, Bromma, Sweden) using the GelScan XL software package (Pharmacia, Uppsala, Sweden). To check for linearity, different amounts of tissue were loaded and the intensity of the cTnI and α-actinin bands was analysed. Protein content of the cardiomyocyte suspension was determined using the RC-DC protein concentration assay kit (BioRad). Based on these determinations protein loads <90 µg were found to be within the linear range.

The cTnI, $cTnI_{1-192}$ and α-actinin bands on gel were identified and the amount of troponin complex exchange was confirmed by Western immunoblotting. Semi-dry blotting was performed at 45 Volt for 90 min, using the discontinue system.[125] Staining with the specific antibodies directed against troponin I (8I-7, Research Diagnostics Inc, dilution 1:10 000) and α-actinin (clone EA53, Sigma, dilution 1:200) was done using the Vectastain ABC-Amp protocol (Vector laboratories, Burlingame, USA).

Microscopy

To check if the cTn complex was homogeneously incorporated into cardiomyocytes, cTn^{DEG} exchanged cardiomyocytes were studied using a Marianas™ digital imaging microscopy workstation equipped with a nanostepper motor (*Z*-axis increments: 10 nm) and

a cooled CCD camera (Cooke Sensicam, Cooke Co, Tonawanda, NY, USA). The microscope, and camera were controlled by SlideBook^{TM} software (SlideBook 4.1.0.9, Intelligent Imaging Innovations, Denver, CO, USA). After the exchange protocol cardiomyocytes were fixed with 4% paraformaldehyde. The $cTnI_{1-192}$ exchange into cardiomyocytes was examined using a specific antibody directed against amino acid residues 195-209 in the C-terminus region of cTnI (clone P45-3, Research Diagnostics Inc, dilution 1:50) and a secondary fluorescent antibody Alexa Fluor 488 (Molecular Probes, dilution 1:40). Images were acquired in the Z-axis direction with a step size of 0.5 μm. Image processing and data analysis was performed using SlideBook^{TM} and ImageJ (public domain software available at http://rsb.info.nih.gov/ij/). Images were deconvoluted using the constrained interactive mode, with interpolation of 1 plane between each acquired plane and mirror edge padding at the Z-axis (20 planes).

Data analysis

Force-pCa relations were fit to a Hill equation:[67]

$$F(Ca^{2+})/F_0=[Ca^{2+}]^{nH}/(Ca_{50}{}^{nH}+[Ca^{2+}]^{nH}),$$

where F is steady state force. F_0 denotes the steady force at saturating Ca^{2+} concentration, nH reflects the steepness of the relation, and Ca_{50} (or pCa_{50}) represents the midpoint of the relation.

Values are given as means ± S.E.M. of n myofibrils of myocytes. Differences were tested by means of unpaired two tailed Student's t-tests at a level of significance of 0.05.

Results

Amount and distribution of exchanged troponin complex in human cardiac preparations

The amount of exchange was analysed in cTn^{DEG} exchanged cardiac samples based on the decline in the ratio between endogenous cTnI and α-actinin using Coomassie stained 1D SDS-PAGE. Figure 4.1A shows an example of a gel of exchanged cardiac myofibrils. Replacement of the endogenous troponin complex with cTn^{DEG} amounted to 54±6% (n=4) in human cardiac myofibrils and to 50±2% (n=5) in human cardiomyocytes. These data were confirmed by Western blots using a specific antibody against cTnI, shown in Figure 4.1B for myofibrils and in Figure 4.1C for cardiomyocytes.

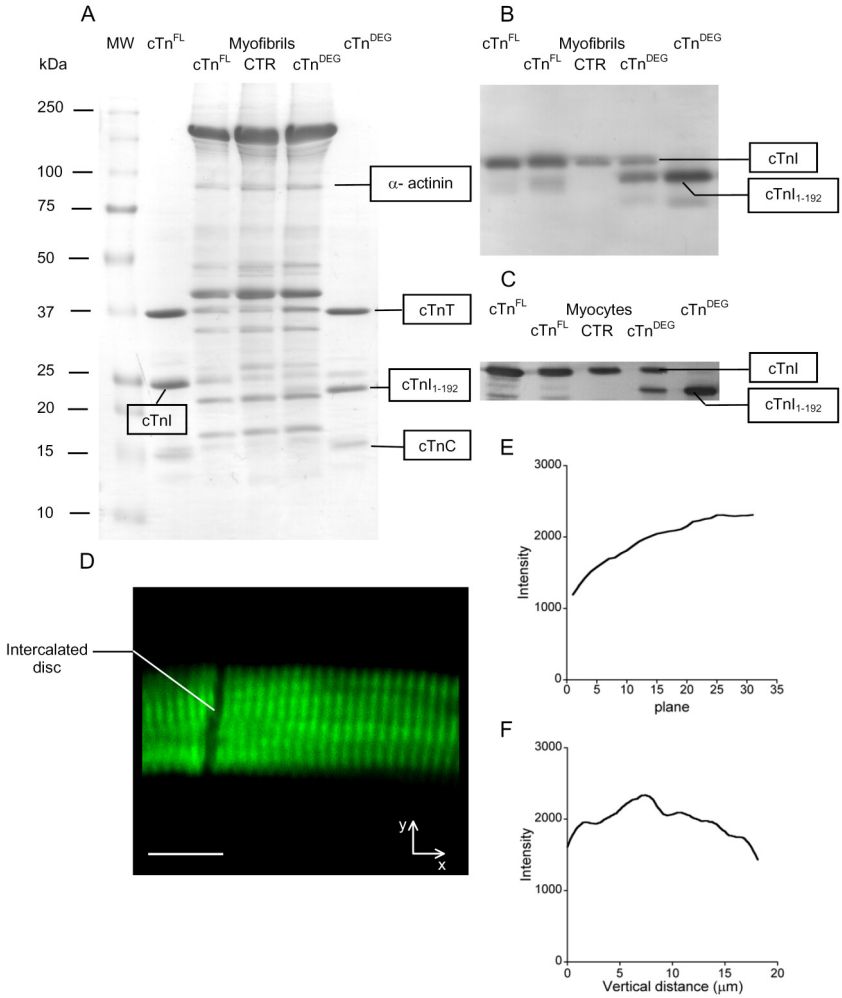

Figure 4.1. **A.** SDS polyacrylamide gel of suspensions of human myofibrils after the exchange procedure with full length cTnI (cTn^{FL}), degraded cTnI (cTn^{DEG}) or without exogenous cTn complex (control condition, CTR). Evaluation of the exchange was performed using the density ratio between endogenous cardiac TnI (cTnI) and α-actinin, as a reference of the amount of protein load. **B.** Subsequent Western immunoblot of a SDS-PAGE gel from human myofibrils using an antibody recognizing the full-length (cTnI) as well as the truncated form ($cTnI_{1-192}$). **C.** Western immunoblot of cTnI in exchanged human cardiomyocytes. **D**. Image obtained by summation of plane intensities along the Z-axis of a single cardiomyocyte exchanged with cTn^{DEG}, incubated with an antibody directed against C-terminus of cTnI. Scale bar: 10 μm. **E.** Distribution of the fluorescence intensity within the cardiomyocyte. Planes were acquired along the Z-axis (in depth) from the top of the myocyte (plane 1) to its bottom (plane 31). **F.** Distribution of the fluorescence intensity along the vertical (y-)axis of cardiomyocyte in its central part (planes 11-21). Abbreviations: cTn^{FL} and cTn^{DEG} denote troponin complex with full-length cTnI and truncated cTnI ($cTnI_{1-192}$), respectively; myofibrils cTn^{FL} and cTn^{DEG} denote myofibrils exchanged with cTn^{FL} and cTn^{DEG}, respectively; CTR denotes myofibrils treated similarly, but without cTnI complexes; MW: molecular weight marker.

To test if the cTn distribution in cardiomyocytes is homogeneous after the exchange, human cardiomyocytes exchanged with cTn^{DEG} were stained with a specific antibody P45-3 and studied using 3D digital imaging microscopy. P45-3 can react only with full-length cTnI and not with truncated $cTnI_{1-192}$, thus staining with this antibody may reveal inhomogeneity of cTn^{DEG} exchange into the cardiomyocytes. A summation in the Z-axis of the X-Y images of a representative cardiomyocyte is shown in Figure 4.1D. Fluorescence intensity was averaged per plane and plotted against plane number in Figure 4.1E. This panel illustrates that with overnight incubation the penetration of cTn into the cardiomyocyte is not limited by diffusion. The fluorescence intensity distribution in the Y-axis direction (Figure 4.1F) in the central part of the myocyte also reveals a rather homogeneous distribution of cTn complex within the exchanged cardiomyocyte. 3D image reconstruction performed in 3 typical cTn^{DEG} exchanged cardiomyocytes showed similar results. This indicates that the protocol used ensures a uniform exchange of cTn^{DEG} inside the cardiomyocytes (and in the much thinner myofibrils).

Force measurements in exchanged human myofibrils

Original recordings of force production in myofibrils at saturating $[Ca^{2+}]$ are shown in Figure 4.2. Maximal isometric force (i.e. force at pCa 3.5 divided by cross-sectional area) was not significantly different between cTn^{FL} and cTn^{DEG} exchanged myofibrils (Figure 4.3A). However, the rate of force rise (K_{ACT}) and the rate of force redevelopment (K_{TR}) were both significantly decreased in cTn^{DEG} exchanged myofibrils compared to cTn^{FL} exchanged myofibrils by 24 and 31%, respectively ($P<0.05$) (Figure 4.3B).

After a rapid switch from activating (pCa 3.5) to relaxing (pCa 9) solution, isometric force started to decline (Figure 4.2). Force relaxation takes place in 2 phases: a slow initial linear phase, with a rate constant $K_{REL,S}$ calculated from its slope, and with a duration t_{SLOW}, and a rapid exponential phase characterized by the rate constant $K_{REL,F}$.[126] The duration of the slow phase t_{SLOW} was significantly increased in cTn^{DEG} exchanged myofibrils (0.30±0.02 s for cTn^{DEG} versus 0.21±0.02 s for cTn^{FL}, $P<0.05$), while $K_{REL,S}$ remained unchanged (0.21±0.02 s^{-1} and 0.31±0.05 s^{-1}, for cTn^{DEG} and cTn^{FL}, respectively). The $K_{REL,F}$ significantly decreased in cTn^{DEG} exchanged myofibrils (2.54±0.25 s^{-1} and 3.75±0.37 s^{-1}, for cTn^{DEG} and cTn^{FL}, respectively, $P<0.05$). An overview of averaged data is presented in Table 4.1.

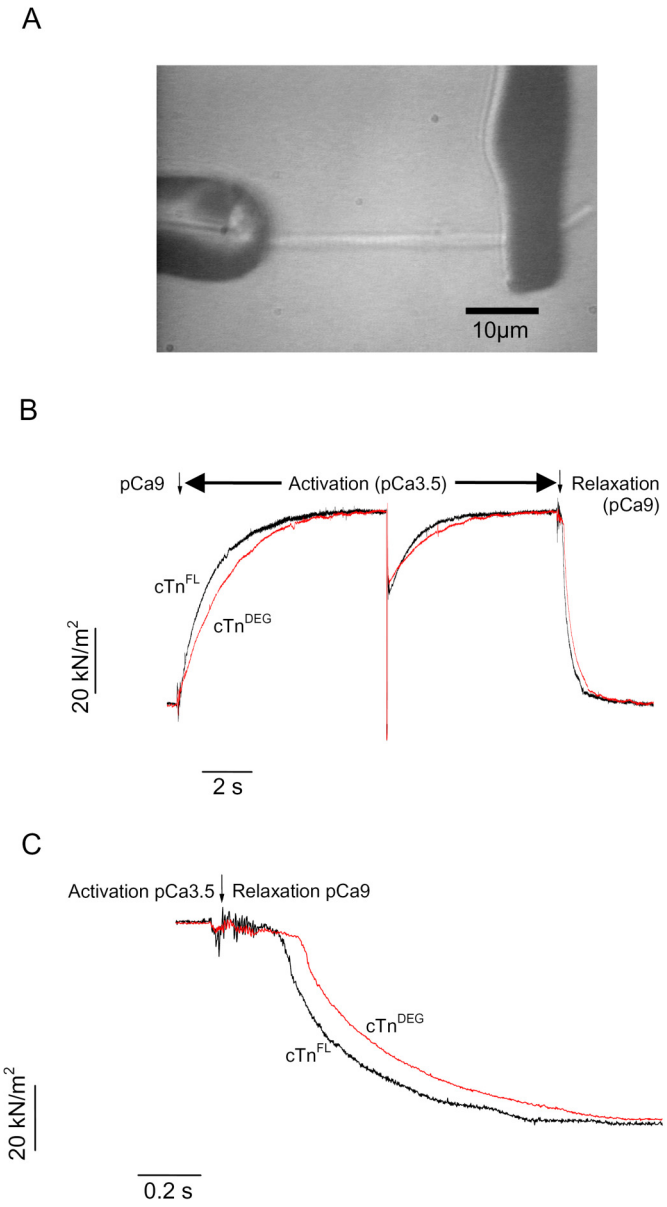

Figure 4.2. **A.** Image of single human cardiac myofibril, **B**. representative traces of force activation and **C.** relaxation in cTn^{FL} and cTn^{DEG} exchanged human cardiac myofibrils. After the myofibril was transferred into activating solution (pCa 3.5) and active force developed the myofibril was shortened by the 20% of its length. Relaxation kinetics were obtained after myofibril was returned into relaxing solution (pCa 9). Scale bar: 10 µm.

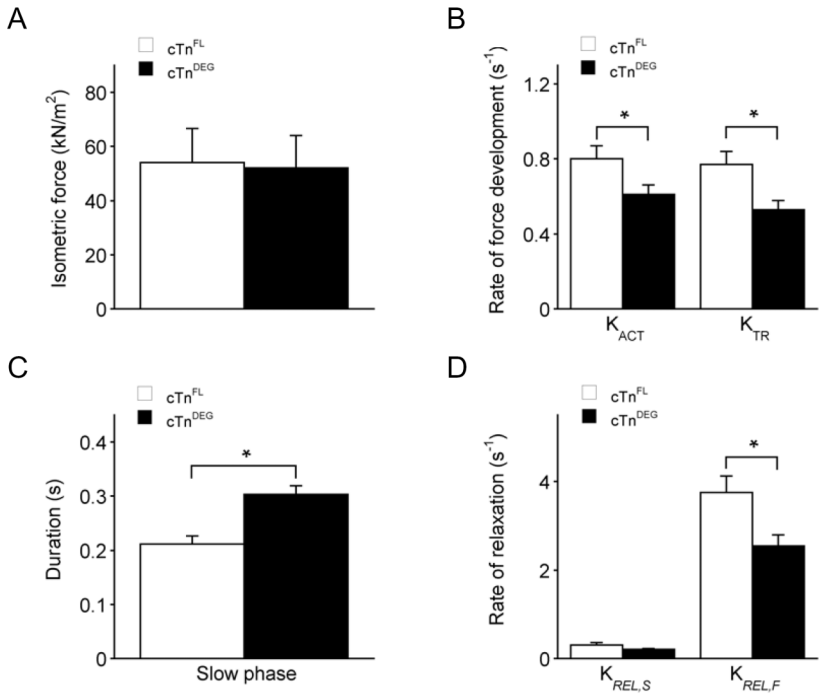

Figure 4.3. **A.** Mean values of maximal isometric force, **B**. rate of force development, **C.** duration of relaxation slow phase and **D.** rate of relaxation of cTn^{FL} or cTn^{DEG} exchanged myofibrils. Maximal force, K_{ACT} and K_{TR} were obtained at maximal [Ca^{2+}] (pCa 4.5). Duration, $K_{REL,S}$, $K_{REL,F}$ were obtained at pCa 9. Values are given as mean ± S.E.M. of 22 cTn^{FL} and 27 cTn^{DEG} exchanged myofibrils. *Significant at p<0.05, cTn^{FL} versus cTn^{DEG} exchanged myofibrils.

Table 4.1. **Functional data of exchanged human myofibrils**

Parameter		**cTn^{FL}**	**cTn^{DEG}**
Force activation	Maximal force ($kN \cdot m^{-2}$)	54±12	52±9
	K_{ACT} (s^{-1})	0.80±0.07	0.61±0.05*
	K_{TR} (s^{-1})	0.77±0.07	0.53±0.05*
Force relaxation	t_{SLOW} (s)	0.21±0.02	0.30±0.02*
	$K_{REL,S}$ (s^{-1})	0.31±0.05	0.21±0.02
	$K_{REL,F}$ (s^{-1})	3.75±0.37	2.54±0.25*

Abbreviations: cTn^{FL} and cTn^{DEG} denote myofibrils exchanged with cTn^{FL} and cTn^{DEG}, respectively. Maximal force, K_{ACT} and K_{TR} were obtained at maximal [Ca^{2+}] (pCa 3.5). t_{SLOW}, $K_{REL,S}$, $K_{REL,F}$ were obtained in relaxing solution (pCa 9). Values are given as mean ± S.E.M. of *n* myofibrils. *Significant at P<0.05, cTn^{DEG} versus cTn^{FL}.

Force measurements in exchanged human cardiomyocytes

The maximal isometric force and its Ca^{2+}-sensitivity could be determined more accurately in cTn^{FL} and cTn^{DEG} cardiomyocytes than in myofibrils, because the cross-sectional area could be determined with a higher precision in cardiomyocytes than in myofibrils (Figure 4.4). Force recordings of cTn^{FL} and cTn^{DEG} exchanged myocytes are presented in Figure 4.5. Maximal isometric force and mean passive force were not significantly different between cTn^{FL} and cTn^{DEG} exchanged myocytes (Figure 4.6). However, cTn^{DEG} cardiomyocytes had a significantly higher Ca^{2+}-sensitivity than cTn^{FL} exchanged cardiomyocytes (pCa_{50}: 5.73±0.03 and 5.52±0.03 for cTn^{DEG} and cTn^{FL}, respectively, $P<0.05$) (Figure 4.7). The cooperativity of force development (nH) tended to decrease in cTn^{DEG} exchanged cardiomyocytes, but the difference between groups did not reach statistical significance (2.08±0.11 and 2.69±0.26 for cTn^{DEG} and cTn^{FL}, respectively, $P=0.05$).

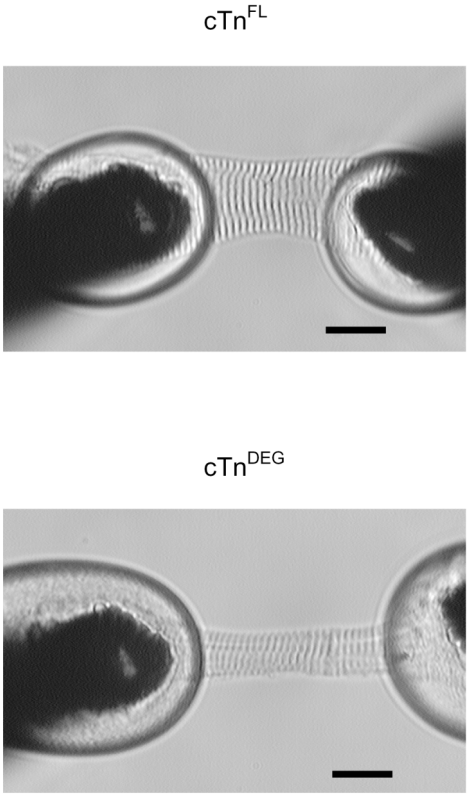

Figure 4.4. **Images of single cardiomyocytes with exchanged cTn^{FL} and cTn^{DEG}.** Scale bar: 25 μm.

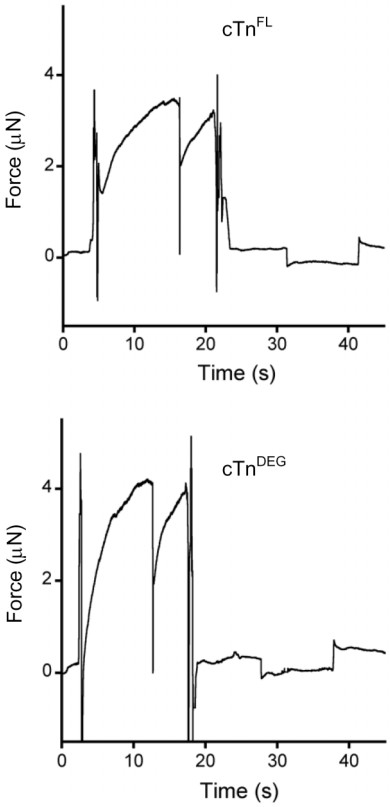

Figure 4.5. **Recordings of force production in cardiomyocytes with exchanged cTn^{FL} and cTn^{DEG} at pCa 4.5.** The myocyte was shortened rapidly after force development reached a steady level to determine the baseline of the force transducer (zero level). After return of the myocyte into the relaxing solution (pCa 9) it was shortened again for 10 s to determine its passive force.

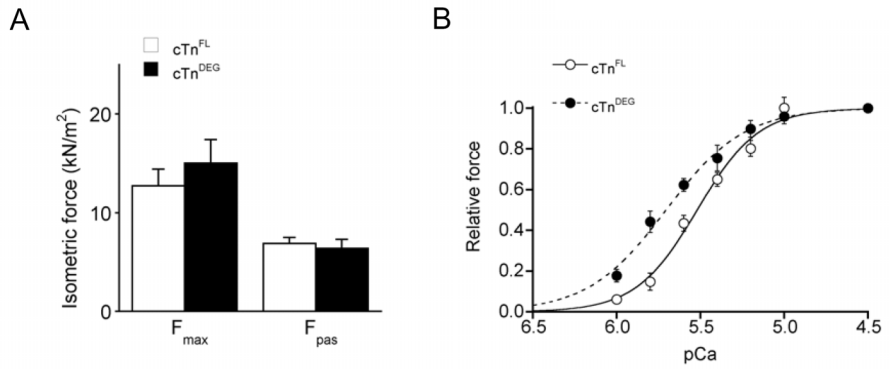

Figure 4.6. **A.** Mean values of maximal isometric force and passive force of exchanged cTn^{FL} and cTn^{DEG} cardiomyocytes. Maximal (F_{max}) and passive (F_{pass}) forces were obtained at saturating [Ca^{2+}] (pCa 4.5) and at low [Ca^{2+}] (pCa 9), respectively. Values are given as mean ± S.E.M. of 14 cardiomyocytes for each group. **B.** The Ca^{2+}-sensitivity of isometric force of exchanged cTn^{FL} and cTn^{DEG} cardiomyocytes. Isometric force at submaximal activating [Ca^{2+}] was normalized to the control force found at saturating [Ca^{2+}] (pCa 4.5). The force-pCa relations are fitted to a Hill equation. Ca^{2+}-sensitivity of cTn^{DEG} cardiomyocytes was significantly higher (pCa_{50}=5.71±0.03) than of cTn^{FL} myocytes (pCa_{50}=5.52±0.02) ($P<0.05$). Values are given as mean ± S.E.M. of 9 cTn^{FL} and 11 cTn^{DEG} exchanged cardiomyocytes.

Discussion

These experiments represent the first attempt to assess the direct effects of the cTnI degradation in human cardiac tissue. The results indicate that cTnI degradation at the C-terminus (1) does not reduce maximal isometric force development nor results in an increase in passive force but (2) slows force development as well as relaxation and (3) enhances myofilament Ca^{2+}-sensitivity.

Comparison with previous studies on $cTnI_{1-192}$ function

The decrease in maximal force generating capacity of the cardiac muscle is one of the main features of reversible ischemia/reperfusion injury (stunning) in rodents and has been attributed to degradation of cTnI, primarily at its C-terminus.[108,109,113] In a transgenic mouse line expressing 9-17% of C-terminus truncated cTnI of total endogenous cTnI,[45,127] maximal force development of intact trabeculae was about 40% depressed compared to non-transgenes. In view of these previous findings it is surprising that in our study the maximum force was not affected in myocytes with the truncated form of TnI. However, it has become increasingly clear that the processes involved in stunning differ in small and large animals.[50] The reduction in force could, at least in part, also be due to degradation of other, ultrastructural proteins[48] or to secondary ROS-related effects.[51] Moreover, species differences may be involved as well.

In a study using an *in vitro* motility assay with reconstituted actin filaments, a 24% decrease in force development was observed of filaments with human $cTnI_{1-192}$ compared to filaments with full-length cTnI.[128] In addition, exchange of the human cTn^{DEG} complex into rabbit psoas myofibrils under the same experimental conditions as used in this study, revealed a 26% decrease in maximal force development in comparison with cTn^{FL} preparations.[123] A similar reduction of maximal force was observed by Tachampa et al.,[129,130] where human or mice cTn complexes were exchanged in rat cardiac trabeculae. The main difference in these exchange experiments is that chimerical thin filaments were formed, whereas in our experiments human cardiac proteins were exchanged into human cardiac tissue. It is conceivable that, despite the high ~95% identity of human, mice, rat and rabbit amino acid sequence of cTn subunits, signal transduction and transmission between troponin subunits and other contractile proteins is isoform and species-dependent. This

might result in diverse functional changes upon $cTnI_{1-192}$ exchange in matching or non-matching species.

In viable remote remodelled tissue after infarction in pigs, minor degradation of cTnI (<4%) was observed while the maximal force generating capacity was reduced.[111] If our findings in humans are characteristic for the situation in large animal models, this would suggest that other factors than TnI degradation would be responsible for this loss in force.

Interestingly, the results on kinetics and Ca^{2+}-sensitivity observed in the present study are in line with those found in exchange experiments using rabbit psoas myofibrils and rat cardiac trabeculae.[123,129,130] However, caution should be exerted with the interpretation of these findings within the context of stunning. For instance Gao et al.[116] have observed an increase in K_{TR} and also provided evidence suggesting that the rate limiting step of the transition from force-generating to non-force-generating states was accelerated in stunned myocardium. Moreover, there is no consensus in the literature regarding the direction of the change in the maximum shortening velocity in stunned myocardium.[131]

Structural and biochemical considerations on $cTnI_{1-192}$ exchange

Recently the atomic structure of human cTn in the Ca^{2+}-saturated form has been resolved using x-ray crystallography.[25] It was concluded that Ca^{2+} binding to cTnC induces detachment of the C-terminus region of cTnI from actin, thereby altering the mobility and/or flexibility of cTn and tropomyosin (TM) on the actin filament. There is good correspondence between this structural model[27] and the three-state biochemical[26] model where tropomyosin can exist in a blocked (B), closed (C) and an open (M) state. In the absence of Ca^{2+}, troponin stabilizes TM in a state that sterically blocks actin interaction with myosin (blocked state). Upon Ca^{2+} binding to cTnC the inhibition is released, and TM adopts a state that allows myosin to bind weakly to actin (closed state). Upon binding of myosin, TM moves further, which allows more and stronger myosin binding to actin, by cooperative propagation along the actin filament (open state). Previous studies indicate that the presence of cTn and TM enhance force development and also influence kinetic parameters of crossbridge interaction.[132-134] This suggests that TnI degradation may affect the maximum force generating capacity as well as the kinetics activation and relaxation of

force. If steric hindrance of the C-terminal end of TnI would be important, as suggested previously,[128] its truncation would tend to increase the number of interacting crossbridges (i.e. force) rather than to decrease it.

The increase in Ca^{2+}-sensitivity can be caused by (1) an increase in Ca^{2+} affinity of cTnC, which promotes the transition from blocked to closed state and/or (2) enhancement of myosin binding to actin, the transition from closed to open state.[20,135] It has been shown using various truncated forms of cTnI that progressive deletion of parts of the C-terminal impairs the ability of cTnI to bind to actin-tropomyosin at low [Ca^{2+}], promoting the availability of actin for binding with myosin.[136] This is consistent with the increase in Ca^{2+}-sensitivity observed in our experiments. The nH of cardiomyocytes exchanged with cTn^{DEG} tended to be lower than that of cTn^{FL} exchanged cardiomyocytes. This may reflect a decrease in cooperativity of thin filament activation, in agreement with the destabilization of TM on actin.

K_{ACT} and K_{TR} of the cTn^{DEG} exchanged cardiomyocytes were both significantly decreased. Evidence suggests that K_{ACT} and K_{TR} are mainly determined by the kinetics of the crossbridge attachment.[28] The parallel changes in K_{ACT} and K_{TR} observed is these experiments are most likely due to cooperative changes within the thin filament that lead to the transition to the open state. Thus, the decreased cooperativity of thin filament regulation may play a role in the slowing of the activation kinetics.

It has been shown that during the first slow linear phase of relaxation all sarcomeres remain isometric, whereas the fast relaxation phase starts when the first mechanically weakest sarcomere 'gives'.[126,137] The slow $K_{REL,S}$ is determined largely by the isoform of myosin heavy chain.[126] This may explain the lack of a significant effect of cTnI degradation on this parameter in our experiments. The duration of the slow phase t_{SLOW} was however increased in cTn^{DEG} exchanged preparations, i.e. sarcomeres remain isometric for a longer time during relaxation. This might be a consequence of the increased Ca^{2+}-sensitivity. Moreover, it has been shown that the fast $K_{REL,F}$ depends inversely on the final steady-state force after relaxation.[138] Since $cTnI_{1\text{-}192}$ incorporation into myofilaments increases Ca^{2+}-sensitivity, the force during relaxation is larger and accordingly the fast $K_{REL,F}$ will be decreased. Thus, changes in both t_{SLOW} and fast $K_{REL,F}$ may be linked to the increased Ca^{2+}-sensitivity.

In a simple two-state model for crossbridge interaction the maximum isometric force is proportional to the number of crossbridges in the force generating state (N), i.e. $f/(f+g)$ and $K_{TR}=f+g$, where f equals the rate of crossbridge attachment and g equals the rate of cross-bridge detachment.[28] If we assume that at saturating Ca^{2+} concentrations $g=K_{REL,S}$, N equals 0.6, both for cTn^{FL} and cTn^{DEG}. Hence, the kinetic values observed are consistent with the preservation of the maximum force-generating capacity.

Implications for the cardiac function in vivo

The amount of cTn exchange into cardiac preparations in our experiments was approximately 50%, while the degree of cTnI degradation observed in failing human myocardium was less (up to 26%).[46] When the concentration of Tn complexes during exchange was doubled, the amount of exchange increased to about 73%. Under these conditions, the decrease in the activation kinetics of cTn^{DEG} exchanged rabbit myofibrils compared to cTn^{FL} exchanged myofibrils was found to be more pronounced (unpublished data). Thus, we expect that the slowing of the kinetics and the increase in Ca^{2+}-sensitivity in failing human myocardium will be less pronounced than observed in our study.

The slowing of relaxation and the increase in Ca^{2+}-sensitivity of cardiac preparations after $cTnI_{1-192}$ exchange observed in the present study suggest that cTnI degradation *in vivo* might impair cardiac relaxation and contribute to diastolic dysfunction. On the other hand, the increased myofilament Ca^{2+}-sensitivity with preserved maximal force generating capacity implies that at Ca^{2+} concentrations present during systole more force will be developed. However, during a twitch this effect will be counteracted by the slowing of force development in preparations with $cTnI_{1-192}$. This will lower the peak force developed during systole suggesting that the net-effect may be rather small. Hence, our data indicate that diastolic dysfunction in human myocardium following (acute) ischemia[139,140] might, at least in part, be due to cTnI proteolysis.

Chapter 5

Exchange of C-terminal truncated troponin I into human cardiac muscle preserves force responses to beta-adrenergic stimulation and increased load

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Submitted combined with Chapter 4.

Abstract

The C-terminal proteolysis of cardiac troponin I (cTnI) has been proposed to play a key role in human ischemic myocardial disease, including stunning. Previously it has been shown that cTnI proteolysis has a large functional effect in human cardiac tissue. In this study the ability of the human myofilaments containing degraded cTnI to modulate their activity in response to changes in cardiac pump function and meet higher hemodynamic demands was investigated. The predominant human cTnI degradation product ($cTnI_{1-192}$) and full length cTnI were expressed in *E. coli*, purified, reconstituted with the other cardiac troponin subunits and subsequently exchanged into permeabilised human cardiomyocytes, isolated from healthy donor hearts. To determine the effect of $cTnI_{1-192}$ on regulation of contractile function, force development was measured in single cardiomyocytes at various calcium concentrations, at sarcomere lengths of 1.9 and 2.2 μm, and after treatment with the catalytic sub-unit of protein kinase A (PKA), to mimic β-adrenergic stimulation. The sarcomere length dependency of force development and the desensitising effect of PKA were preserved in $cTnI_{1-192}$ exchanged cardiomyocytes. These results indicate that degradation of cTnI in human myocardium does not affect β-adrenergic and preload-dependent responses.

Introduction

A detrimental role of cardiac troponin I (cTnI) degradation has been implicated in human ischemic heart disease. [38,45,46] Degradation of cTnI was observed in viable cardiac tissue of patients who required surgery for ischemic disease [45,46] and transplant hearts of patients diagnosed with an extensive coronary artery disease. [38] Strong evidence for involvement of cTnI C-terminal degradation in reversible ischemia/reperfusion injury (i.e. stunning) is arising from animal studies.[45,108,109] Recently, we have shown that exchange of $cTnI_{1-192}$ (which corresponds to the primary degradation product observed in human cardiac ischemic tissue) into human cardiac myofibrils and cardiomyocytes results in a substantial slowing of force generation and relaxation kinetics, an increase of Ca^{2+}-sensitivity, but has no effect on maximal force (Chapter 4). These results indicate that cTnI proteolysis found in ischemic

heart disease may impair diastolic function and contribute to the development of heart failure in humans.

Cardiac TnI has a central position in the signaling pathway connecting Ca^{2+}-binding to the troponin complex (cTn) to myosin binding to actin. Recently, structural data obtained by X-ray crystallography[25] revealed that the N-terminus of cTnI binds to cardiac troponin C (cTnC) and cardiac troponin T (cTnT), while its central inhibitory region (IR) and C-terminal domain in the absence of Ca^{2+} are attached to the actin filament (Chapter 1, Figure 1.3). These interactions with tropomyosin (TM) and actin impede myosin binding to actin. When Ca^{2+} binds to cTnC, both the IR and C-terminal domain move away from the tropomyosin and actin and allow myosin binding to actin. Thus, the loss of either IR or C-terminal domain of cTnI would be expected to alter the mechanism of Ca^{2+} regulation of muscle contraction and substantially affect the pump function of the human heart.

Cardiac TnI is not only essential for Ca^{2+}-regulation of cardiac myofilament activation, but also is important in the adjustment of myofilament activity to changes in hemodynamic demands. Prominent mechanisms by which cardiac myofilament activation process is coupled to the prevailing hemodynamic state and beating frequency include (1) sympathetic nervous system, through protein kinase/phosphatase pathways and (de)phosphorylation of regulatory proteins, and (2) ventricular filling (preload): the Frank-Starling mechanism.[141]

Regulation by sympathetic nervous system involves β-adrenergic receptor stimulation with subsequent phosphorylation of cTnI by protein kinase A (PKA) at its N-terminus (Ser 23 and 24). Phosphorylation of cTnI by the catalytic subunit of PKA causes a decrease in Ca^{2+}-sensitivity of isometric force production in human cardiomyocytes.[39,142,143] It has been shown by measurements of Ca^{2+}-controlled thin filament– activated myosin S1-ATPase activity and *in vitro* motility assays that a mutation in the cTnI C-terminus (K206Q), known to cause familial hypertrophic cardiomyopathy, reduces the effect of PKA treatment.[144] This suggests that cTnI C-terminal domain composition is important in PKA signal transduction.

The dependence of ventricular systolic pressure on ventricular volume (as a result of diastolic filling) is commonly known as the Frank-Starling law of the heart. The cellular basis underlying the Frank-Starling mechanism is the increase in contractile force with an increase in sarcomere length. This occurs due to the change in the overlap of actin and

myosin and an increase in Ca^{2+}-sensitivity of force development.[145,146] The molecular mechanisms underlying the length-dependent alteration of Ca^{2+}-sensitivity are still obscure. However, it has been shown that the increase in Ca^{2+}-sensitivity associated with the increase in sarcomere length was significantly blunted in cardiac myofilaments dissected from transgenic mice expressing slow skeletal troponin I.[147] This suggests that cTnI is an important determinant of the length-dependent activation of cardiac myofilaments.

The aim of this study was to elucidate whether or not the human myofilaments containing degraded cTnI would be able to modulate their activity in response to changes in cardiac pump function and meet higher hemodynamic demands. More specifically, the question was studied whether partial degradation of cTnI from its C-terminus has such a big impact on its structure that it would alter PKA-induced phosphorylation of cTnI as well as length-dependence of activation.[16]

To address this question force measurements at maximal and submaximal [Ca^{2+}] were performed in human cardiomyocytes, in which endogenous troponin complexes were replaced by complexes containing either human full-length cTnI or truncated $cTnI_{1-192}$. The force measurements were performed at a sarcomere length of 1.9 and 2.2 μm and before and after PKA treatment. The results indicated that $cTnI_{1-192}$ did not affect the length dependency of force activation and the effect of PKA-induced phosphorylation of human cardiac myocytes.

Materials and Methods

Preparation of recombinant troponin complex, isolation of human cardiomyocytes and exchange of cardiac Tn complex into cardiomyocytes were performed as described in Chapter 4.

Force measurements in exchanged human myofibrils and cardiomyocytes

Isometric force in single human cardiomyocytes was measured at different [Ca^{2+}] (pCa= -log [Ca^{2+}] ranging from 4.5 to 6.0) and at a sarcomere length of 1.9 and 2.2 μm as described previously.[60,142] After attachment the cardiomyocyte was stretched to a sarcomere length of 1.9 μm. After an initial maximal activation 6 measurements were carried out at submaximal [Ca^{2+}] (pCa>4.5) followed by maximal activation. Force values obtained in solutions with

submaximal [Ca^{2+}] were normalized to the interpolated maximal force values (pCa 4.5). The decrease in maximal force during this first force-pCa series amounted to 2±1% for cardiomyocytes containing cTn^{FL} and 2±3% for cardiomyocytes containing cTn^{DEG}. Thereafter the cardiomyocyte was stretched to a sarcomere length of 2.2 μm, and force measurements at maximal and submaximal [Ca^{2+}] were repeated. After the full force-pCa curve was obtained, the cardiomyocyte was incubated for 40 min at 20°C in relaxing solution containing 6 mmol/l dithiothreitol (DTT) and the catalytic subunit of PKA (3 mg/l [100 U/ml]; Sigma, batch 35H9522). After incubation the force-pCa series at 2.2 μm was repeated. On average force decline between the first and the last maximal control activation in theses series of force-pCa measurements amounted to 10±2% for cTn^{FL} and 11±3% for cTn^{DEG} exchanged cardiomyocytes before and to 15±2% for cTn^{FL} and 12±2% for cTn^{DEG} exchanged cardiomyocytes after treatment with PKA. Experiments where force decline exceeded 25% were discarded. As a result not all the myocytes included in this study were measured at all 3 experimental conditions (at sarcomere length 1.9 μm, 2.2 μm and 2.2 μm after the PKA). All the results obtained were divided in 2 groups: sarcomere length (n=10 for cTn^{FL} and n=12 for cTn^{DEG} exchanged cardiomyocytes) and PKA treatment (n=10 for cTn^{FL} and n=13 for cTn^{DEG} exchanged cardiomyocytes) groups, each of which contained paired (1.9/2.2 μm and before/after PKA) measurements on the same myocytes. Maximal activation at 2.2 μm and pCa 4.5 was used to calculate maximal calcium-activated isometric force (i.e. F_{max}/cross-sectional area). Maximal isometric force at sarcomere length 1.9 μm was normalized to the cross-sectional area of the cardiomyocyte obtained at sarcomere length 2.2 μm. The cross-sectional area of the preparations was calculated assuming an elliptical shape, i.e. cross-sectional area = (width × depth × π)/ 4. Myocyte depth was estimated by projecting the myocyte via a small mirror, positioned at an angle of 30°, onto the objective of the inverted microscope.[122]

Protein analysis

The amount of cTnI exchange was analysed as described previously in Chapter 4.

Data analysis

Force-pCa relations were fit to a Hill equation:[67]

$$F(Ca^{2+})/F_0=[Ca^{2+}]^{nH}/(Ca_{50}{}^{nH}+[Ca^{2+}]^{nH}),$$

where F is steady state force at 1.9 and 2.2 μm sarcomere length. F_0 denotes the steady force at saturating Ca^{2+} concentration, nH reflects the steepness of the relation, and Ca_{50} (or pCa_{50}) represents the midpoint of the relation.

Values are given as means ± S.E.M. of *n* myofibrils of myocytes. Results were stated to be significant based on two tailed Student's unpaired *t*-tests at a level of significance of 0.05.

Results

Effect of sarcomere length on cardiomyocyte function

Recordings of force development and images of cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes at two different sarcomere lengths are presented in Figure 5.1 and 5.2. Mean dimensions (± S.E.M.) of the cTn^{FL} (*n*=10) and cTn^{DEG} (*n*=12) exchanged cardiomyocytes in sarcomere length group at a sarcomere length of 1.9 μm amounted to 36.4±7.8 and 40.3±4.7 μm in length, 26.5±2.2 and 28.1±2.3 μm in width, respectively. After the force-pCa curve was determined the cardiomyocyte was stretched to 2.2 μm sarcomere length. At this sarcomere length mean dimensions of the cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes amounted to 55.4±9.4 and 62.6±6.4 μm in length, 23.7±2.4 and 24.3±2.5 μm in width, and 20.5±1.3 and 24.2±1.6 μm in depth, respectively. At neither sarcomere lengths the dimensions of the cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes were significantly different. An overview of functional data is presented in Table 5.1. Maximal isometric force (measured at pCa 4.5) and mean passive force of both cTn^{FL} and cTn^{DEG} exchanged myocytes were increased at sarcomere length 2.2 μm compared to values obtained at a sarcomere length of 1.9 μm (Figure 5.3A). In addition, the Ca^{2+}-sensitivity of cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes was significantly higher at higher sarcomere length (pCa_{50}: 5.44±0.02 and 5.54±0.02 for cTn^{FL} and 5.68±0.02 and 5.81±0.02 for cTn^{DEG}, at 1.9 and 2.2 μm respectively, $P<0.05$) (Figure 5.3B). The cooperativity of force development (nH) was found to be unchanged by increasing sarcomere length (Table 5.1).

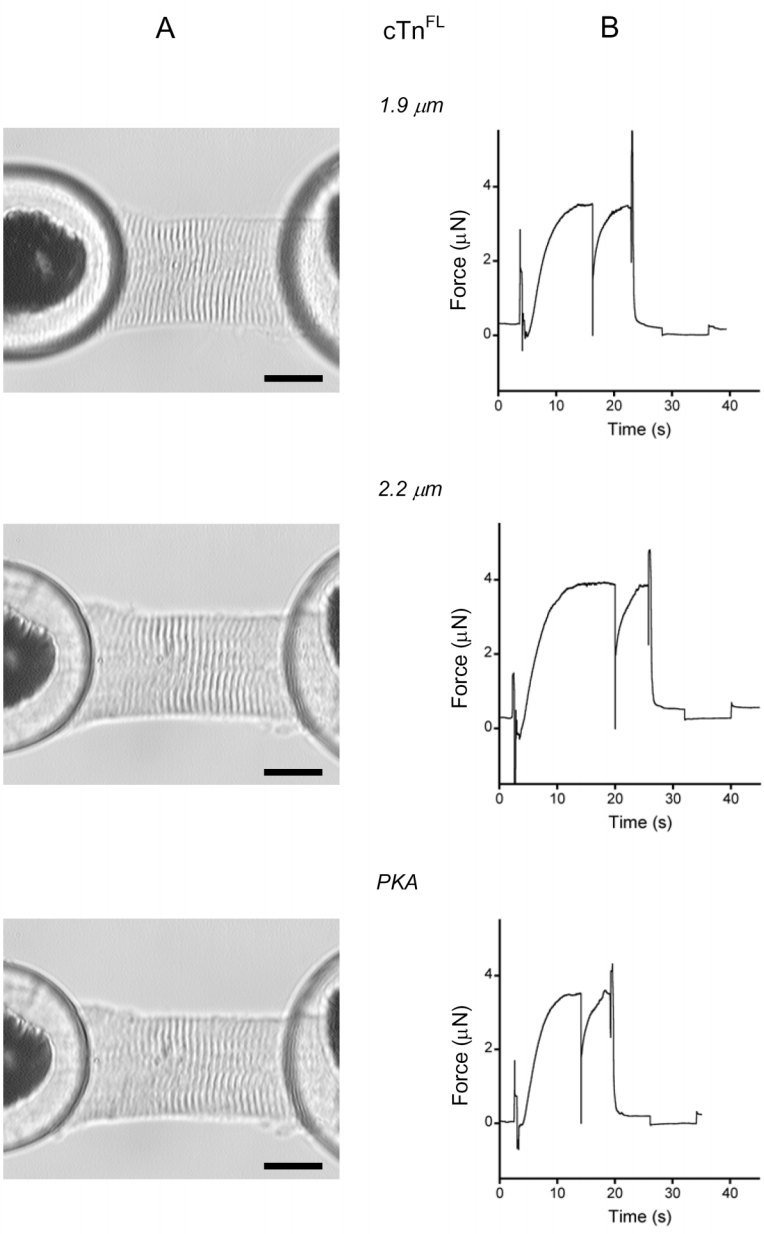

Figure 5.1. **A.** Images of single cardiomyocytes with exchanged cTn^{FL} at sarcomere length 1.9 µm, 2.2 µm and after treatment with PKA (at sarcomere length 2.2 µm). The bars indicate 25 µm. **B.** Corresponding recordings of force production in cardiomyocytes with exchanged cTn^{FL} at pCa 4.5. The myocyte was slacked rapidly after force development reached a steady level to determine the baseline of the force transducer (zero level). After return of the myocyte into the relaxing solution (pCa 9) it was slacked again for 10 s to determine its passive force.

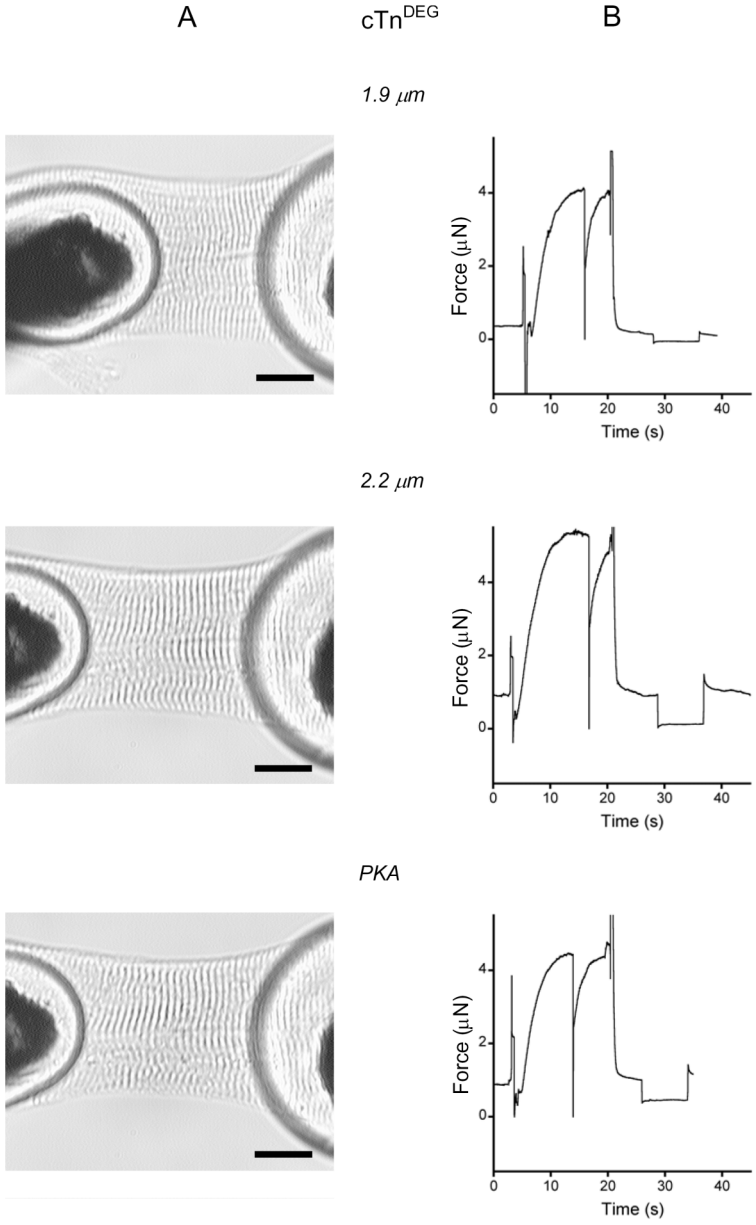

Figure 5.2. **A.** Images of single cardiomyocytes with exchanged cTn^{DEG} at sarcomere length 1.9 µm, 2.2 µm and after treatment with PKA (at sarcomere length 2.2 µm) and **B.** Corresponding recordings of force production in the same cardiomyocytes at pCa 4.5. For detailed description see legend of Figure 5.1.

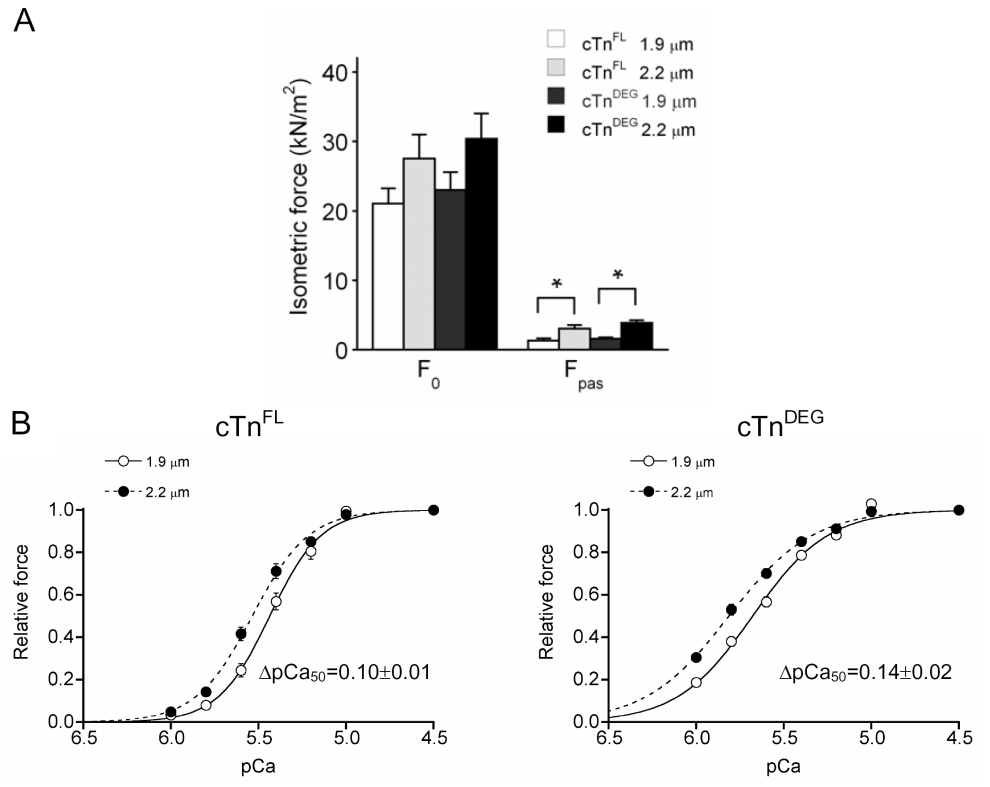

Figure 5.3. **A.** Mean values of maximal isometric force and passive force of exchanged cTn^{FL} and cTn^{DEG} cardiomyocyte at sarcomere lengths 1.9 and 2.2 μm. Maximal (F_0) and passive (F_{pass}) forces were obtained at saturating [Ca^{2+}] (pCa 4.5) and at low [Ca^{2+}] (pCa 9), respectively. F_0 and F_{pass} at sarcomere length 2.2 μm of both cTn^{FL} and cTn^{DEG} cardiomyocyte were significantly higher than at 1.9 μm. Values are given as mean ± S.E.M. of 10 cTn^{FL} cardiomyocytes and 12 cTn^{DEG} cardiomyocytes in each group. **B.** The Ca^{2+}-sensitivity of isometric force of exchanged cTn^{FL} cardiomyocytes at sarcomere lengths 1.9 (open symbols) and 2.2 (closed symbols) μm. Isometric force at submaximal activating [Ca^{2+}] was normalized to the control force found at saturating [Ca^{2+}] (pCa 4.5). The force-pCa relations are fitted to a Hill equation. ΔpCa_{50} was calculated by subtraction of pCa_{50} obtained at 1.9 μm from pCa_{50} obtained at 2.2 μm for each individual cardiomyocyte and then averaging this value per group. Ca^{2+}-sensitivity of cTn^{FL} cardiomyocytes at 2.2 μm was significantly higher than at 1.9 μm ($P<0.05$). **C.** The Ca^{2+}-sensitivity of isometric force of exchanged cTn^{DEG} cardiomyocytes at sarcomere lengths 1.9 and 2.2 μm. cTn^{DEG} cardiomyocytes at 2.2 μm were more sensitive to [Ca^{2+}] than at 1.9 μm ($P<0.05$). Values are given as mean ± S.E.M.

Table 5.1. **Functional data of exchanged human cardiomyocytes**

Group	Parameter	Sarcomere length		PKA treatment	
		1.9 µm	2.2 µm	before	after
cTn^{FL} *n=10/10*	Maximal force ($kN \cdot m^{-2}$)	21±2	28±3	34±5	34±4
	Passive force ($kN \cdot m^{-2}$)	1.32± 0.33	3.04±0.51*	3.48±0.49	2.58±0.24
	pCa_{50}	5.44±0.02	5.54±0.02*	5.52±0.02	5.44±0.01*
	nH	3.14±0.09	2.88±0.15	2.87±0.15	2.96±0.17
cTn^{DEG} *n=12/13*	Maximal force ($kN \cdot m^{-2}$)	23±3	30±4	30±3	28±3
	Passive force ($kN \cdot m^{-2}$)	1.60±0.17	3.87±0.41*	3.53±0.47	2.77±0.27
	pCa_{50}	5.68±0.02$^{\#}$	5.81±0.02*$^{\#}$	5.78±0.02$^{\#}$	5.69±0.02*$^{\#}$
	nH	2.07±0.08$^{\#}$	1.88±0.06$^{\#}$	1.94±0.07$^{\#}$	2.13±0.08$^{\#}$

Abbreviations: cTn^{FL} and cTn^{DEG} denote cardiomyocytes exchanged with cTn^{FL} and cTn^{DEG}, respectively. *n* denote number of cardiomyocytes in sarcomere length/PKA- treated groups, respectively. Values are given as mean ± S.E.M. of *n* cardiomyocytes. *Significant at P<0.05, within groups 1.9 - 2.2 µm and before versus after PKA; # significant at P<0.05, cTn^{DEG} versus cTn^{FL}.

Effect of PKA on cardiomyocyte function

Original force recordings and images of cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes before and after treatment with PKA are shown in Figure 5.1 and 5.2. In the PKA treatment group the mean dimensions of the cTn^{FL} (n=10) and cTn^{DEG} (n=13) exchanged cardiomyocytes were 50.9±9.4 and 59.6±5.4 µm in length, 20.7±2.2 and 24±2 µm in width, and 18.9±1.4 and 22.5±1.3 µm in depth, respectively. Maximal isometric force and mean passive force were not different in cTn^{FL} and cTn^{DEG} exchanged myocytes (Table 5.1, Figure 5.4A). In both groups treatment with PKA resulted in a decrease in Ca^{2+}-sensitivity (Figure 5.4B). The pCa_{50} values in the cTn^{FL} group amounted to 5.52±0.02 and 5.44±0.01 and the pCa_{50} in the cTn^{DEG} group amounted to 5.78±0.02 and 5.69±0.02 before and after the PKA treatment, respectively, P<0.05. As can be seen in Table 5.1 the nH of cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes did not change after PKA treatment.

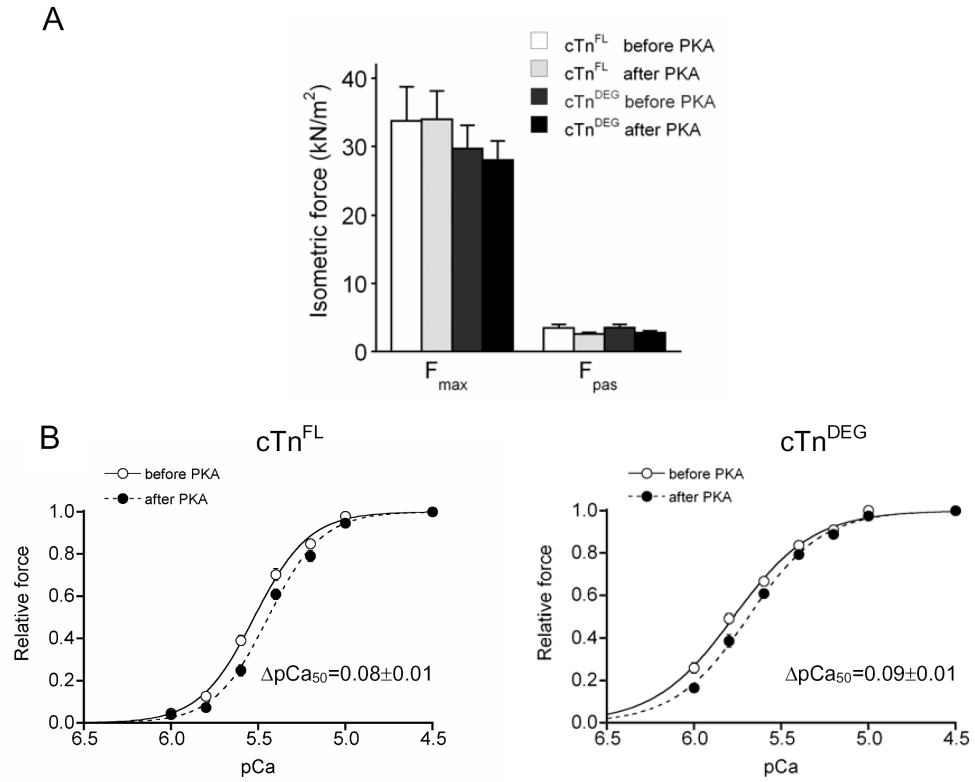

Figure 5.4. **A.** Mean values of maximal isometric force and passive force of exchanged cTn^{FL} and cTn^{DEG} cardiomyocytes before and after the treatment with PKA at 2.2 µm sarcomere length. Maximal (F_{max}) and passive (F_{pass}) forces were obtained at saturating [Ca^{2+}] (pCa 4.5) and at low [Ca^{2+}] (pCa 9), respectively. Values are given as mean ± S.E.M. of 10 cTn^{FL} cardiomyocytes and 13 cTn^{DEG} cardiomyocytes in each group. **B.** The Ca^{2+}-sensitivity of isometric force of exchanged cTn^{FL} cardiomyocytes before (open symbols) and after (closed symbols) the PKA treatment. Isometric force at submaximal activating [Ca^{2+}] was normalized to the control force found at saturating [Ca^{2+}] (pCa 4.5). The force-pCa relations are fitted to a Hill equation. For description of ΔpCa_{50} see Figure 5.3. Ca^{2+}-sensitivity of cTn^{FL} cardiomyocytes after the PKA treatment was significantly lower ($P<0.05$). **C.** The Ca^{2+}-sensitivity of isometric force of exchanged cTn^{DEG} cardiomyocytes before and after the PKA treatment. cTn^{DEG} cardiomyocytes after the PKA treatment were less sensitive to [Ca^{2+}] than before that ($P<0.05$). Values are given as mean ± S.E.M.

cTn^{DEG} versus cTn^{FL} exchanged cardiomyocytes

At both sarcomere lengths (1.9 and 2.2 μm) and before and after phosphorylation by PKA the maximal isometric force and mean passive force were not significantly different between cTn^{FL} and cTn^{DEG} exchanged myocytes (Table 5.1). In contrast, a significantly higher Ca^{2+}-responsiveness of force generation was observed in cTn^{DEG} cardiomyocytes compared to cTn^{FL} at both sarcomere lengths. The difference in Ca^{2+}-responsiveness observed between cTn^{FL} and cTn^{DEG} exchanged cardiomyocytes did not differ upon increasing sarcomere length (ΔpCa_{50}=0.24±0.03 and ΔpCa_{50}=0.27±0.03 at 1.9 and 2.2 μm, respectively, P=0.5). This difference was also preserved upon phosphorylation with PKA at 2.2 μm (ΔpCa_{50}=0.26±0.03 and ΔpCa_{50}=0.25±0.02 before and after PKA treatment, respectively, P=0.8). The cooperativity of force development (nH) was found to be significantly decreased in cTn^{DEG} exchanged cardiomyocytes compared to cTn^{FL} at all conditions (Table 5.1).

Amount of exchanged troponin complex in human cardiomyocytes

The analysis of $cTnI_{1\text{-}192}$ incorporation into cardiomyocytes by Western blots using a specific antibody against cTnI showed that 48% of the endogenous troponin complex was replaced with cTn^{DEG}. These data confirmed our previous results described in Chapter 4.

Discussion

The results of this study indicate that cTnI degradation at the C-terminus preserves both sarcomere length-dependency and protein kinase A phosphorylation signalling in human cardiomyocytes. More specifically, cTnI degradation did not affect (1) the increase in maximal force, mean passive force and the Ca^{2+}-sensitivity of the contractile apparatus with an increase in sarcomere length from 1.9 to 2.2 μm and (2) the desensitisation to Ca^{2+} as a result of treatment with PKA. Thus, the ability to modulate its activity in response to changes in hemodynamic demands of the heart is preserved in human cardiac tissue containing degraded cTnI.

The present data at a sarcomere length of 2.2 μm confirm our previous results (Chapter 4) on the effects of cTnI C-terminal truncation on human cardiac myofilament function. However, the maximal forces in this study were almost twice as large as in the

previous study. This difference could be due to the differences in tissue handling and storage (samples used in this study were stored in liquid nitrogen, while samples from the previous study were kept at -80°C). It is important to note that the absence of an effect on maximal force and the similar magnitude of the Ca^{2+}-sensitivity shift found in cTn^{DEG} exchanged cardiomyocytes compared to cTn^{FL} preparations found in both studies suggest that the observed effects can be attributed solely to the degradation of cTnI and not to intrinsic properties of human cardiac tissue.

Sarcomere –length dependency of myofilament activation

The exact mechanism of increased Ca^{2+}-responsiveness due to the increase in sarcomere length is not completely clear yet. It has been shown that affinity of cTnC to Ca^{2+} is increased with length.[148,149] However, more recent experiments have shown that cTnC is less likely to be a crucial component in the length sensing mechanism.[150] Previously, length-tension relationships were found to be much steeper in skinned cardiac than in skinned skeletal muscles and this was explained by different TnC isoforms in these tissues.[151-153] In these recent experiments transgenic mice were developed in which fast skeletal TnC was expressed ectopically in the heart.[150] A comparison of the length dependence of activation between myocytes with endogenous cTnC and myocytes containing primarily fast skeletal TnC revealed no difference. From this it was concluded that the sarcomere length dependent change in Ca^{2+}-sensitivity of tension is independent of the troponin C isoform.

Another hypothesis is that a change in sarcomere length, when volume remains constant, results in a change in diameter and in interfilament spacing that modulates the ability of crossbridges to react with the thin filament at equal Ca^{2+} concentrations.[146] McDonald et al.[154] proposed that the likely mechanism for the change in force with interfilament spacing is the change in the crossbridges transition rate from weak to strong binding states (f_{app}). This change would affect the number of strongly bound force-generating crossbridges and thus, the twitch tension at a given concentration of Ca^{2+}. However, recently it has been shown that the alterations in myofilament lattice spacing may not be the sole mechanism that underlies the sarcomere length induced alteration of Ca^{2+}-sensitivity in myocardium.[103] Moreover, the cooperativity of myofilament activation, which

can be affected by the changes in filament lattice spacing, has been shown to be also unchanged during the sarcomere length alterations.[155]

Thin-filament proteins have been suggested to be involved in the mechanism for length dependent activation.[141] Previously it has been shown in a transgenic mouse model that specific isoform switching from cTnI to slow skeletal TnI in thin myofilaments reduced length-dependent activation of cardiac myofilaments.[147] Thus, the absence of an effect of cTnI degradation on sarcomere length dependency observed in this study is surprising. The ability of degraded cTnI to preserve the length-dependent activation of cardiac myofilaments and preserve the magnitude of the difference between Ca^{2+}-sensitivity of cTn^{FL} and cTn^{DEG} cardiomyocytes at different sarcomere lengths suggests that the C-terminal domain of cTnI is not involved in the mechanism of length dependent activation. Even though this part of cTnI contains actin-tropomyosin binding sites,[25] the distribution of blocked, closed and open crossbridges at different sarcomere lengths apparently is not regulated by the C-domain. A significant structural difference between cTnI and slow skeletal TnI is that the cardiac variant possesses an additional 30 aminoacids forming an N-terminal extension not present in the slow skeletal variant. This could be the reason for the observed attenuation of the length dependence of Ca^{2+} activation in preparations containing slow skeletal TnI.[147] However the involvement of other protein changes, which may develop in transgenic animals, can not be excluded.

The fibres from transgenic mouse hearts expressing slow skeletal TnI was found to be more sensitive to Ca^{2+} compared to wild-type controls.[147] It has been suggested though that this can not be the reason for the smaller increase of Ca^{2+}-sensitivity with an increase in sarcomere length in these preparations. This conclusion was made based on the experiments, in which specific isoform switching from α-TM to β-TM in the hearts of transgenic mice induced a sensitisation of the cardiac myofilaments to Ca^{2+}, but did not alter the length dependent activation.[156] Our data are in agreement with this conclusion: differences in cooperativity and Ca^{2+}-sensitivity between cTn^{DEG} and cTn^{FL} cardiomyocytes were observed, but length-dependent activation was preserved.

Evidence suggests that other proteins can determine the magnitude of shift in Ca^{2+}-sensitivity due to the increase in sarcomere length. The different myosin isoforms and/or other proteins (such as e.g. titin,[157,158] myosin-binding protein C[159,160] and myosin light chain 2[161]) may account for length-dependent activation rather than cTnI.

Effect of phosphorylation by PKA on myofilament activation

β-Adrenergic stimulation is a major physiological mechanism to meet increases in circulatory demand, which acts through enhancement of systolic function as well as relaxation.[162] Activation of PKA via this signaling pathway leads to phosphorylation of a several proteins within muscle cells. In skinned preparations all the membranous structures (plasma membrane, sarcoplasmatic reticulum) are removed, which leaves only three targets for PKA, myosin binding protein C (MyBP-C), cTnI and titin. It has been shown that PKA phosphorylation of cTnI reduces the myofilament Ca^{2+}-sensitivity,[62,142] increases Ca^{2+}-dissociation from cTnC[163] and increases the crossbridge cycling rate and shortening velocity,[164-167] while the maximum rate of ATP consumption remained constant.[62] In this study a decrease in Ca^{2+}-sensitivity of cTn^{FL} exchanged cardiomyocytes upon PKA was observed, which is in agreement with our previous data obtained in human cardiac tissue.[142,143]

Results of NMR structural studies[168,169] and cross-linking studies[170] support a mechanism in which phosphorylation of PKA sites of cTnI (Ser 23/24) triggers dissociation of the N-terminal region of cTnI from cTnC, inducing a reduction in affinity of cTnC for Ca^{2+} and a decrease in Ca^{2+} -sensitivity of the myofilaments. It has been shown that phosphorylation of Ser 23/24 causes cTnI to become more compact.[171] Moreover, fluorescence resonance transfer revealed that phosphorylation of these serine sites causes a decrease in the mean distance between the N- and C-terminal regions of cTnI[172] and an increase in helicity of the backbone structure.[144] It was hypothesized that this conformational change involves bending of the cTnI N-terminus such that Ser23/24 phosphorylation destabilizes the interaction between the N-terminus of cTnI and the regulatory region of cTnC[173] and this in turn probably destabilizes Ca^{2+}-binding to cTnC.

The C-terminus of cTnI (residue 206 in particularly) was shown to be important for the structural responsiveness of cTnI upon phosphorylation.[144] Thus, the preservation of PKA induced desensitisation of myofilaments observed in this study was somewhat unexpected. The explanation for this might be the existence of structural alterations inside the troponin complex in cardiac myofilament, which compensate for the loss of the cTnI C-terminus and preserve its function.

Implications for the in vivo function

Our results suggest that β-adrenergic responsiveness and length-dependency of the cardiomyocytes is not affected by C-terminal truncation of cTnI. The amount of exchange in our setting was about 50%, while the highest degree of cTnI degradation found in humans is about 26%.[46] This suggests that the effect of $cTnI_{1-192}$ *in vivo* will be less compared to what was found here. However, even though cTn complex of patients with ischemic cardiomyopathy will be able to modulate the heart function upon an increase in hemodynamic demands (such as during physical exercise or stress), the Ca^{2+}-sensitivity of filament contraction in these hearts will be still increased compared to healthy cardiac tissue. This might still contribute to the diastolic dysfunction and ultimately heart failure in these patients.

It is tempting to speculate that selective C-terminal proteolysis of cTnI induces a feed-forward activation loop in the progression of ischemia related human cardiomyopathies (Figure 5.6).

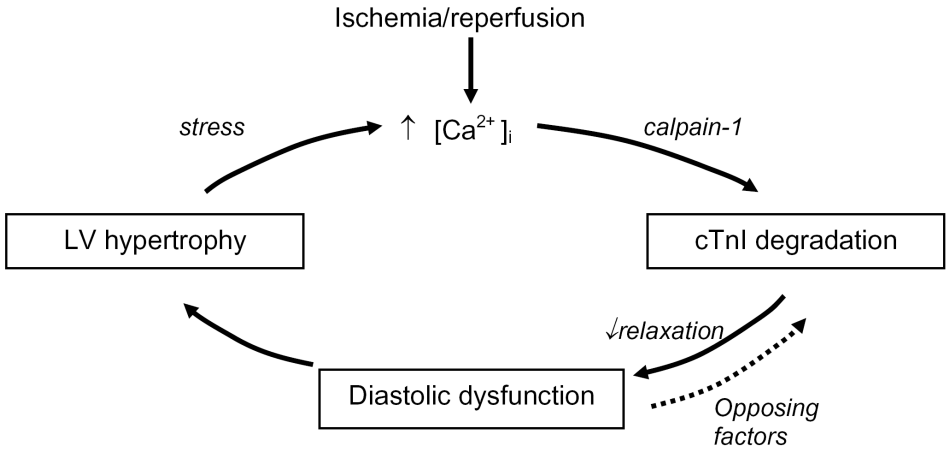

Figure 5.6. **Role of cTnI degradation in induction and development of heart failure after ischemia.**

Ca^{2+} overload induced by ischemia/reperfusion leads to an activation of the Ca^{2+}-dependent protease calpain-1. It has been shown that this protease induces specific C-terminus degradation of cTnI.[109] The $cTnI_{1-192}$ alters cardiac myofilament activation by an increase of Ca^{2+}-sensitivity and by slowing both activation and relaxation kinetics (Chapter 4). This results in prolonged relaxation of human myocardium and leads to diastolic

dysfunction, which in turn may cause left ventricular hypertrophy.[92,174,175] The contractility of myocardium can be modulated by β-adrenergic signaling pathway or increased preload (denoted as *opposing factors* in Figure 5.6), however it will not be able to completely compensate for the loss of muscle contractility. It has been shown that mechanical stress increases cytosolic Ca^{2+} levels in neonatal rat cardiomyocytes[176] and this may induce further activation of calpain-1. Certainly, there are many more cellular processes involved in the development of ischemic cardiomyopathy. They were not mentioned here to highlight this particular component within a more general framework. This scheme specifically points out the role of cTnI degradation in the development of ischemic cardiomyopathy and may explain how chronic depletion of cTnI from viable remodelled myocardium after myocardial infarction may lead to a global contractile dysfunction as observed in pigs.[111] However, the relative contribution of cTnI selective proteolysis into induction and progression of human ischemic cardiomyopathy remains to be established.

Chapter 6

Summary and conclusions

Summary

Alterations of contractile proteins occur in human heart failure. They include both changes in protein expression (translational modifications) and modulation by phosphorylation and degradation of the proteins (post-translational modifications). These changes may have a beneficial effect in order to maintain pump function of diseased myocardium. They can also be detrimental and substantially contribute to the progression of contractile dysfunction of failing human heart. Hence, a new therapeutic strategy should take into account the functional consequences of compensatory and/or detrimental contractile protein changes that occur during heart failure. Therefore, it is of a great importance to reveal contractile protein alterations in human heart failure and their influence on the functional properties of human cardiac tissue.

In this thesis, mechanical and energetic consequences of the contractile protein changes, which occur during heart failure were studied in human healthy and failing myocardial tissue. Isometric force development and ATP consumption at different calcium concentrations were measured in chemically skinned atrial trabeculae and ventricular muscle strips to reveal the effect of MHC isoform changes. The effect of cTnI degradation on isometric force development and force activation and relaxation kinetics was studied using cardiac myofibrils and cardiomyocytes. Manipulation of troponin composition in human cardiac preparations was achieved by replacement of endogenous cTn complex by an exogenous troponin complex containing truncated cTnI. Contractile protein isoform composition was determined by one-dimensional SDS-gel electrophoresis, while phosphorylation status of proteins was obtained using specific phospho-antibodies in Western immunoblotting. Protein composition was correlated with functional properties of human cardiac tissue to determine if and to what extent protein changes are beneficial or detrimental for human cardiac pump function.

In **Chapter 1**, an introduction to the background and methods of this study is given. The aim of this research is defined.

In **Chapter 2**, the relation between contractile properties and MHC isoform composition in healthy human myocardium was determined. One-dimensional SDS-PAGE revealed that in ventricular tissue the β-MHC isoform predominated, whereas in atrial tissue a variable mixture of α- and β-MHC was found. Force development in ventricular

tissue was about 5-fold more economical, but 9 times slower, than in atrial tissue. Significant linear correlations were found between MHC isoform composition, ATP consumption and rate of force redevelopment. These correlations suggest that the increased economy and decreased speed of contraction found in ventricular tissue as compared to atrial tissue can be attributed to the prevalence in this tissue of the β-MHC isoform.

The rate of ATP consumption of preparations with pure (100%) α-MHC was approximately 5 times higher than the rate in preparations with pure β-MHC (0.181±0.018 and 0.039±0.014 $mmol \cdot l^{-1} \cdot s^{-1}$, respectively, for extrapolated values ± their standard errors). The K_{TR} associated with pure α-MHC expression calculated from the regression line (10.8 s^{-1}) was considerably higher than the values obtained in ventricular tissue (0.9 s^{-1}). These results indicate that even a small shift in MHC isoform expression may have a considerable impact on cardiac performance in human tissue.

Histochemical analysis revealed that the average density of myofibrillar tissue was significantly lower in atrial than in ventricular preparations (50±9 and 89±2%, respectively). The difference in the percentage of interstitial space in atrial (20±4%) and ventricular (11±2%) tissue did not reach statistical significance (P=0.09). The correction of the data for the proportion of myofibrillar tissue abolished the difference in maximal tension, increased the difference in ATPase activity and slightly enhanced the relative difference in the extrapolated ATPase-values of the "pure" isoforms. It also slightly improved the correlation between ATPase activity and β-MHC composition. This difference in the average density of myofibrillar tissue between atrial and ventricular preparations and atrial intra-trabeculae variability should be taken into consideration in studies, where these preparations are used.

In **Chapter 3**, the relation between functional properties and MHC isoform composition in diseased human myocardium was studied. Chronic atrial fibrillation was accompanied by a significant shift from the fast α-MHC isoform to the slow β-MHC isoform compared to the MHC isoform composition in tissue from patients with sinus rhythm (β-MHC content shifted from 24.6±3.2 to 38.1±5.7% of total MHC). On the other hand, both donor and end-stage failing ventricular tissue contained almost exclusively β-MHC isoform: only in two donors and one failing heart sample the α-MHC isoform was detected. The economy of contraction and other functional properties were all preserved in

atrial fibrillation and in end-stage human heart failure. However, Ca^{2+}-sensitivity of ATP consumption was significantly higher in AF than in SR patients, while Ca^{2+}-sensitivity of force did not differ. This implies that tension cost of atrial contraction in AF patients decreases with an increase in Ca^{2+} concentration. In failing ventricular tissue Ca^{2+}-sensitivity of both force and ATP consumption were increased compared to non-failing donors.

The increase in β-MHC content of healthy and diseased cardiac tissue correlated with a decrease in ATP consumption and in tension cost. From these correlations it appears that the shift in MHC composition towards the β-MHC isoform may be beneficial under pathological conditions, since less energy is required to maintain pump function. Because human ventricular tissue mainly consists of β-MHC, this shift is of less importance in ventricles, but it is a prominent feature of failing atria.

Histochemical analysis revealed the percentage of interstitial space (mainly occupied by connective tissue) in both non-failing atrial and ventricular tissue was lower than in diseased tissue. Thus development of fibrosis can also contribute to reduced pump function of failing human myocardium (Chapter 1, Figure 1.1).

In **Chapter 4**, the functional implications of cTnI degradation were investigated in human cardiac tissue. Endogenous cTnI was replaced by the primary degradation product of cTnI ($cTnI_{1\text{-}192}$) or by full length cTnI in both cardiac myofibrils and cardiomyocytes. Maximal tension was not affected, while the rates of force activation and redevelopment as well as relaxation kinetics were slowed in the presence of truncated cTnI. Ca^{2+}-sensitivity of the contractile apparatus was increased in preparations containing $cTnI_{1\text{-}192}$ complex compared to full-length exchanged cardiomyocytes. These results indicate that degradation of cTnI in human myocardium will mainly impair diastolic function.

In **Chapter 5**, the effect of cTnI degradation on myofilament responsiveness to β-adrenergic stimulation and to increased preload (Frank-Starling effect) was investigated in human cardiomyocytes. PKA-mediated desensitisation and sarcomere length dependent increase in force-generating capacity of myofilaments were preserved in $cTnI_{1\text{-}192}$ exchanged cardiomyocytes. This indicates that cTnI degradation does not affect β-adrenergic and preload-dependent responses in human myocardium.

Conclusions

1. Myocardial contraction is 5-fold more economical in ventricular than in atrial human tissue.

The 5-fold difference in economy of contraction results from decreased ATP consumption of ventricular tissue, since no significant difference in isometric force per cross-sectional area was observed between atrial and ventricular preparations. This functional difference should be taken into account during extrapolation of results obtained on ventricular tissue to the properties of atrial tissue and vice versa.

2. Human β-MHC isoform is 5 times more economical, but considerably slower than α-MHC isoform.

From the correlation between the rate of ATP consumption and MHC composition, the rate of ATP consumption of fibres with pure α-MHC and pure β-MHC isoforms could be calculated. The decreased tension cost value of fibres with 100% β-MHC isoform results from a decreased rate of ATP consumption, since no correlation between isometric force per cross-sectional area and β-MHC isoform content was observed.

3. Under pathological conditions the shift in MHC composition towards the β-MHC isoform may be beneficial in human atrial tissue, but plays little or no role in ventricular tissue.

Since β-MHC isoform is more economical than α-MHC, the increased content of β-MHC in cardiac muscle fibres will be beneficial for failing human heart, because less energy will be required to maintain pump function. It should be noted however that under severe cardiovascular stress the MHC isoform shift might become disadvantageous since cardiac reserve required at this conditions largely determined by the rate of myocardial contraction, which will be considerably slowed with the increased content of β-MHC. These effects are mostly characteristic to atrial tissue, since healthy atrial tissue contains only about 25% of β-MHC. Hence, the MHC isoform shift observed during cardiac disease (e.g. atrial fibrillation) will exert a large functional effect. Human ventricular tissue mainly consists of β-MHC (up to 100%). Thus the MHC isoform shift, which may occur in some individuals,

will be considerably smaller than in atria and hence will have less effect on myocardial pump function.

4. The direct effect of changes in troponin composition on functional properties of human cardiac tissue can be assessed by exchange of modified troponin complex into cardiac human preparations.

As it has been shown with digital imaging microscopy, the protocol used for the exchange of troponin complex into human cardiomyocytes ensures its uniform exchange into the cardiomyocytes. At the given conditions the exchanged cardiomyocytes develop isometric force comparable to the non-exchanged cardiomyocytes and remain stable during the application of a whole range of sub-maximal calcium activations. This makes the exchange an excellent technique to study the specific functional effect of both translational and post-translational modifications of cardiac troponin subunits in human cardiac tissue.

5. Degradation of cTnI from its C-terminus may contribute to diastolic dysfunction in human myocardium.

Maximal tension was not affected after the exchange of $cTnI_{1\text{-}192}$ containing troponin complexes into human cardiac myofibrils and cardiomyocytes, which indicated that systolic function was not affected by degradation of cTnI. The slowing of relaxation and activation kinetics and increased Ca^{2+}-sensitivity of the contractile apparatus in preparations containing $cTnI_{1\text{-}192}$ indicates that degradation of cTnI might rather impair diastolic function and in this way contribute to development of heart failure in humans.

6. Cardiac TnI degradation does not affect β-adrenergic and preload-dependent responses in human myocardium.

PKA-mediated desensitisation and sarcomere length dependent increases in force-generating capacity of the myofilaments were preserved in the $cTnI_{1\text{-}192}$ exchanged cardiomyocytes. This indicates that the response to cardiovascular stress (e.g. as observed during exercise) will not be blunted in human hearts containing degraded cTnI.

In brief, in this thesis it has been shown to what extent MHCs determine the energetic properties of human cardiac tissue and cTnI is shown to play a pivotal role in determining its Ca^{2+}-sensitivity.

Future directions

Oxidation of contractile proteins

Oxidative stress has been assigned an important role in reducing myocardial function during heart failure.[177,178] *Oxidation* is another type of myofilament protein post-translational modification, and represents a new topic in the research on altered myofilament function in diseased human heart. The major reactive oxygen species (ROS) and their derivatives, reactive nitrogen species (RNS) are superoxide radicals ($O_2^{-\bullet}$), hydroperoxyl radicals ($HO_2^{\bullet}$), nitric oxide (NO) and peroxynitrite ($ONOO^-$). Collectively, these radicals cause a loss of biological function of contractile proteins through oxidation of the protein backbone and/or amino acid side chains, which may lead to protein fragmentation and the formation of the protein-protein cross-linkages, respectively. Recent evidence suggests an important role for oxidative stress during atrial fibrillation in humans.[93,179] Mihm et al.[93] showed an increased prevalence of contractile protein oxidation, nitration and carbonyl formation in patients with AF. The important role of myocardial NAD(P)H oxidase activation and NO-synthase uncoupling has been demonstrated in patients with paroxysmal and permanent AF.[179]

In addition, detrimental consequences of contractile protein oxidation have been implicated in ischemia/reperfusion injury.[180,181] Some time ago ROS–induced damage to myofibrillar proteins has been proposed as a important mechanism underlying contractile dysfunction observed during myocardial stunning.[182] However, only recently a start has been made in the identification of contractile proteins affected by oxidation. It has been shown in rat hearts that actin undergoes the major changes as a result of postischemic reperfusion.[180] In an experimental model of coronary microembolization in dogs and pigs, the contractile dysfunction was related to reversible oxidation of tropomyosin.[181] Moreover, it has been shown that addition of superoxide anion or peroxynitrite to cardiac muscle preparations reduced or even completely abolished maximal calcium-activated force via post-translational modification of myofilament proteins.[183-185]

These studies point towards an important role for contractile protein oxidation in cardiac dysfunction. Hence, prevention of protein oxidation may preserve or even increase myocardial contractility in human heart failure. The use of general antioxidants in multiple clinical trials did not prove to be beneficial.[186] However, a selective approach directed against specific target proteins and specific ROS/RNS radicals may demonstrate positive results. Thus, future research on the effect of myofibrillar protein oxidation on functional properties of human failing myocardium is very promising.

Future treatment approaches of heart failure

Heart failure imposes a high risk of repeated hospitalisation and death in the human population. Despite the clear benefit of current therapies the mortality in this syndrome remains high. Therefore the search for new therapies has a high priority.

New targeted therapies that would increase or maintain contractility by selective modulation of specific target proteins represent an important research area. One possible therapy is to alter the expression of proteins using gene transfer. Recently it has been shown that expression of the βARKct (a peptide inhibitor of the β-adrenergic receptor kinase) via adenovirus-mediated gene transfer can improve contractile function and β-adrenergic responsiveness in failing human myocytes.[187] Also, it has been shown that adenoviral gene transfer of antisense phospholamban (asPL), which decreases the inhibitory effects of phospholamban on SERCA2a can improve contractile function in failing human myocardium.[188] Unlike inotropic agents that improve contractile function at the expense of increased mortality and worsening metabolism it has been shown that gene transfer of SERCA2a improves survival and the energy potential in failing rat hearts.[189] Thus, targeting of specific myofilament proteins may provide therapeutic benefits in human heart failure.

In the first part of this thesis it has been shown that increased expression of β-MHC in human heart failure improves the myofilament energetics and therefore may be of great help for the cardiac pump function. Whether it would be beneficial to induce a further shift in MHC composition is doubtful. Since at the same time it would significantly decrease the speed of myocardial contraction, the response to cardiovascular stress (e.g. physical activity) in patients would become severely impaired. Recently this has been

demonstrated in transgenic mice with almost complete replacement of the normally predominant α-MHC isoform with β-MHC.[85]

Apart from reversal of translational protein changes, post-translational alterations might be easy to target with therapy and should become a primary focus for the development of new interventional strategies. The effect of cTnI C-terminal degradation investigated in the last part of this thesis is clearly detrimental. Therapies directed on reduction of the cTnI proteolysis may improve the depressed contractility after ischemia and prevent development of heart failure in humans. The first indication that inhibition of calpain-1 (the protease, which induces cTnI degradation) indeed reduces contractile dysfunction upon ischemia/reperfusion injury has been obtained in rats.[190] An alternative approach to prevent cTnI degradation is via modulation of its phosphorylation status. *In vitro* studies have shown that protein kinase A-mediated cTnI phosphorylation protects cTnI from degradation,[108] while phosphorylation by protein kinase C enhances degradation.[191] Investigation of the effect of commonly used medication, which could increase cTnI phosphorylation by PKA (β-blockers) and decrease phosphorylation by PKC (ACE-inhibitors) on recovery of myocardial pump function is currently performed within a collaborative project in a pig model of myocardial infarction (Laboratory for Physiology, VUmc, Amsterdam & Experimental Cardiology, Thoraxcenter and Department of Biochemistry, Erasmus MC, Rotterdam). Further experiments are required to test the feasibility of both these approaches in human myocardium.

The questions addressed in this thesis provide an insight into the cellular pathomechanisms of depressed contractility in human heart failure. The information about the role of MHC isoforms and cTnI obtained in these studies is important for the development of new protein targeted therapeutic interventions, which will improve treatment and decrease mortality of heart failure in humans.

Chapter 7

Samenvatting en conclusies

Samenvatting

Hartfalen wordt bij mensen onder meer gekenmerkt door veranderingen in contractiele eiwitten van de hartspier. Deze veranderingen kunnen zowel optreden op het niveau van eiwitexpressie (translationele modificatie) als op het niveau van modulatie, door middel van fosforylering en degradatie van eiwitten (post-translationele modificatie). Deze processen kunnen een gunstig effect hebben op de functie van het falende hart, maar soms dragen ze juist bij aan de progressie van de contractiele disfunctie. Het is erg belangrijk om meer te weten te komen over de functionele consequenties van compensatoire en/of schadelijke veranderingen in de contractiele eiwitten tijdens de ontwikkeling van hartfalen, zodat met deze kennis nieuwe therapeutische strategieën ontwikkeld kunnen worden.

In dit proefschrift worden in gezond en falend humaan hartspierweefsel de mechanische en energetische consequenties bestudeerd van veranderingen in contractiele eiwitten die optreden tijdens hartfalen. Stukjes spierweefsel afkomstig van de kamer en trabekels, afkomstig van de boezem zijn chemisch ontdaan van hun membraan. De isometrische contractiekracht en ATP-consumptie is gemeten bij verschillende calciumconcentraties, om zo het effect te bepalen van veranderingen in de isoform van de zware keten van myosine (MZK; myosin heavy chain, MHC). In myofibrillen en cardiomyocyten is het effect van degradatie van troponine I (cTnI) op isometrische contractiekracht, krachtsactivatiekinetiek en krachtrelaxatiekinetiek ook bestudeerd. In deze studie werd de samenstelling van het troponine in de humane hartspierpreparaten gemanipuleerd door het endogene troponine-complex te vervangen door een exogeen complex dat getrunceerd troponine bevatte. De isoform van het contractiele eiwit werd bepaald met behulp van eendimensionale SDS-gelelectroforese, en voor het bepalen van de mate van fosforylering van de eiwitten werd gebruik gemaakt van specifieke fosfo-antilichamen in een Western blot. De eiwitsamenstelling werd gecorreleerd met de functionele eigenschappen van het humane hartspierweefsel, om zo te bepalen of – en in welke mate – veranderingen in deze eiwitten een gunstig of schadelijk effect hebben op de pompfunctie van het hart.

Hoofdstuk 1 bevat een introductie over de achtergrond en de methoden van deze studie. Het doel van dit onderzoek wordt gedefinieerd.

In **Hoofdstuk 2** wordt de relatie tussen contractiele eiwitten en MZK isoform compositie in gezond humaan hartspierweefsel bepaald. Eendimensionale SDS-PAGE toonde aan dat in weefsel afkomstig van de hartkamer vooral β-MZK voorkomt, terwijl weefsel afkomstig van de boezems verschillende verhoudingen van zowel α- als β-MZK bevat. De krachtsontwikkeling in kamerweefsel was ongeveer vijf keer meer economisch, maar negen keer trager dan in boezemweefsel. Er bestonden significante lineaire correlaties tussen MZK isoform compositie, ATP consumptie, en de tijd van herontwikkelen van de kracht (K_{TR}). Dit suggereert dat de toegenomen economie en afgenomen snelheid van de contractie in het spierweefsel van de hartkamer ten opzichte van die in de boezems, toegeschreven kan worden aan de hogere prevalentie van de β-isoform in de kamers.

De ATP-consumptie van preparaten die voor 100% uit α-MZK bestonden was ongeveer vijf keer hoger dan die van preparaten die alleen β-MZK bevatten (0.181±0.018 and 0.039±0.014 $mmol \cdot l^{-1} \cdot s^{-1}$, respectievelijk, voor geëxtrapoleerde waarden ± hun standaardfouten). De K_{TR} geassocieerd met pure α-MZK expressie berekend van de regressielijn (10.8 s^{-1}) was aanzienlijk hoger dan de waarden verkregen in kamerweefsel (0.9 s^{-1}). Dit betekent dat zelfs een kleine verschuiving in MZK isoform expressie aan aanzienlijke impact kan hebben op het functioneren van het humane hart.

Histochemische analyse toonde aan dat de gemiddelde dichtheid van myofibrillair weefsel significant lager was in boezemweefsel dan in kamerweefsel (50±9 and 89±2%, respectievelijk). Het verschil in het percentage interstitiële ruimte in boezem- (20±4%) en kamer- (11±2%) weefsel was niet statistisch significant (p=0.09). Na correctie van de data voor de proportie myofibrillair weefsel verdween het verschil in maximale tensie, nam het verschil in ATPase activiteit toe, en nam het relatieve verschil in de geëxtrapoleerde ATPase-waarden van de “pure” isovormen licht toe. Ook verbeterde de correlatie tussen ATPase activiteit en β-MZK compositie. Met dit verschil in gemiddelde dichtheid van myofibrillair weefsel tussen preparaten afkomstig uit boezem- en kamerweefsel, evenals met de variabiliteit in atriale trabekels, zou rekening moeten worden gehouden in studies waarbij van deze preparaten gebruik wordt gemaakt.

In **Hoofdstuk 3** is de relatie tussen functionele eigenschappen en MZK isoform compositie in ziek humaan hartweefsel bestudeerd. Chronisch boezemfibrilleren ging gepaard met een significante verschuiving van de snelle α-isoform naar de trage β-isoform,

vergeleken met de MZK isoform compositie in weefsel van patiënten met sinusritme (het percentage β-MZK verschoof van 24.6±3.2 naar 38.1±5.7% van het totale MZK). Zowel kamerweefsel van donoren als kamerweefsel van patiënten met eind-stadium hartfalen bevatte daarentegen vrijwel uitsluitend β-MZK: de α-MZK isoform was slechts in twee donorharten en een falend hart aantoonbaar. De economie van de contractie evenals de overige functionele eigenschappen waren niet afgenomen bij boezemfibrilleren en eind-stadium hartfalen. De Ca^{2+}-gevoeligheid van de ATP-consumptie was daarentegen significant hoger in patiënten met boezemfibrilleren dan in mensen met sinusritme, terwijl de Ca^{2+}-gevoeligheid van de kracht niet verschillend was. De tension cost (ATP verbruik gedeeld door de kracht) van de boezemcontractie in patiënten met boezemfibrilleren neemt dus af met een toename van de Ca^{2+} concentratie. In kamerweefsel van patiënten met hartfalen zijn de Ca^{2+}-gevoeligheid van zowel kracht als ATP-consumptie toegenomen vergeleken met donoren zonder hartfalen.

De toename in β-MZK in gezond en ziek hartspierweefsel correleerde met een afname van de ATP-consumptie en tension cost. Uit deze correlaties kan men opmaken dat de verschuiving in β-MZK isoform gunstig is onder pathologische omstandigheden, aangezien hij leidt tot een afname van de benodigde energie voor het behoud van de pompfunctie. Deze verschuiving is minder belangrijk in kamers, omdat humaan kamerweefsel vooral uit β-MZK bestaat, maar dit proces is wel een prominent kenmerk van falende boezems.

Histochemische analyse toonde aan dat het percentage interstitiële ruimte (die vooral bindweefsel bevat) in niet-falend boezem- en kamerweefsel lager was dan in ziek weefsel. De ontwikkeling van fibrose kan dus ook bijdragen aan de afgenomen pompfunctie van falend humaan hartspierweefsel (Hoofdstuk 1, Figuur 1.1).

In **Hoofdstuk 4** zijn de functionele gevolgen van cTnI degradatie in humaan hartweefsel bestudeerd. In zowel myofibrillen als in cardiomyocyten werd endogeen cTnI vervangen door het primaire degradatieproduct van cTnI ($cTnI_{1-192}$), of door compleet cTnI. Dit had geen invloed op de maximale tensie, terwijl de snelheid van krachtsactivatie en K_{TR}, evenals de relaxatie, trager waren in de aanwezigheid van getrunceerd cTnI. De Ca^{2+}-gevoeligheid van het contractiele apparaat was toegenomen in preparaten die $cTnI_{1-192}$ bevatten, vergeleken met cardiomyocyten waarin endogeen cTnI was uitgewisseld tegen

compleet cTnI. Dit betekent dat cTnI degradatie in humaan hartspierweefsel vooral een schadelijke invloed zal hebben op de diastolische functie van het hart.

Het effect van cTnI degradatie op de gevoeligheid van myofilamenten voor β-adrenerge stimulatie en een toegenomen voorbelasting (het Frank-Starling effect) in humane cardiomyocyten wordt beschreven in **Hoofdstuk 5**. PKA-gemedieerde desensitisatie en sarcomeerlengte-afhankelijke toename in de kracht-opwekkende capaciteit van myofilamenten waren niet afgenomen in cardiomyocyten waarin endogeen cTnI was uitgewisseld tegen $cTnI_{1\text{-}192}$. Dit betekent dat cTnI degradatie geen invloed heeft op β-adrenerge en voorbelasting-afhankelijke responsen van humaan hartspierweefsel.

Conclusies

1. In humane harten is de contractie van de hartspier vijfmaal meer economisch in kamerweefsel dan in boezemweefsel.

Het vijfvoudige verschil in economie van de contractie is het gevolg van een afgenomen ATP consumptie in kamerweefsel, aangezien tussen boezem- en kamerpreparaten geen significant verschil werd gezien in de isometrische kracht gecorrigeerd voor cross-sectioneel oppervlak. Dit functionele verschil moet in aanmerking worden genomen als resultaten verkregen in kamerweefsel worden geëxtrapoleerd naar boezemweefsel, of vice versa.

2. De humane β-MZK isoform is vijfmaal meer economisch, maar aanzienlijk trager dan de α-MZK isoform.

Uit de correlatie tussen de mate van ATP-consumptie en MZK-compositie kon de mate van ATP-consumptie van vezels met alleen α-MZK en alleen β-MZK isovormen worden berekend. De afgenomen tension cost waarde van vezels met 100% β-MZK is het gevolg van een afgenomen ATP-consumptie, aangezien geen correlatie tussen de isometrische kracht gecorregeerd voor cross-sectioneel oppervlak en de hoeveelheid β-MZK werd gezien.

3. Onder pathologische omstandigheden lijkt de verschuiving in MZK samenstelling naar de β-MZK isoform gunstig te zijn in boezemweefsel, maar deze speelt hooguit een kleine rol in kamerweefsel.

Aangezien de β-MZK isoform meer economisch is dan de α-isoform, zal een toename van de hoeveelheid β-MZK in hartspiervezels gunstig zijn voor een falend humaan hart, omdat minder energie nodig zal zijn voor het in stand houden van de pompfunctie. Hierbij moet echter wel opgemerkt worden dat de MZK isoform verschuiving ongunstig kan worden ten tijde van zware cardiovasculaire belasting. De cardiale reserve die in deze omstandigheden nodig is, hangt namelijk in belangrijke mate af van de snelheid van de contractie, en deze neemt aanzienlijk af bij een toename van de hoeveelheid β-MZK. Deze effecten treden vooral op in boezemweefsel, aangezien gezond boezemweefsel slechts 25% β-MZK bevat. De MZK isoform verschuiving die wordt gezien bij hartziekten (bijvoorbeeld boezemfibrilleren) zal in de boezems dus een groot functioneel effect hebben. Humaan kamerweefsel bestaat vooral uit β-MZK (tot 100%). De MZK isoform verschuiving die in sommige patiënten zal optreden zal in de kamers dus aanzienlijk kleiner zijn, en daardoor zal het effect op de pompfunctie van het hart beperkt zijn.

4. Het directe effect van de samenstelling van troponine op de functionele eigenschappen van humaan hartweefsel kan worden bepaald door gemodificeerd troponine complex uit te wisselen tegen "endogeen" troponine in humane hartspierpreparaten.

Digital imaging microscopy liet zien dat de uitwisseling van een "exogeen" troponine complex tegen endogeen troponine in de hartspiercellen uniform was. Onder de geldende condities ontwikkelen de uitgewisselde hartspiercellen een isometrische kracht die vergelijkbaar is met niet-uitgewisselde hartspiercellen, en blijven de cellen stabiel gedurende het toedienen van een uitgebreide serie sub-maximale calcium-activaties. Dit maakt deze uitwisselingstechniek uitermate geschikt om de specifieke functionele effecten van zowel translationele als post-translationele modificaties van cardiale troponine subunits te bestuderen in humaan hartweefsel.

5. Degradatie van cTnI aan de C-terminale zijde draagt mogelijk bij aan diastolische dysfunctie in het humane hart.

Uitwisseling van $cTnI_{1-192}$-bevattende troponine complexen in humane cardiale myofibrillen en hartspiercellen had geen invloed op de maximale spanning. Dit duidt erop dat de systolische functie niet beïnvloed werd door cTnI degradatie. De vertraagde relaxatie en activatiekinetica, evenals de toegenomen Ca^{2+}-gevoeligheid van het contractiele apparaat in $cTnI_{1-192}$-bevattende preparaten wijst er op dat cTnI degradatie eerder een schadelijke invloed heeft op de diastolische functie, en op die manier een bijdrage levert in de ontwikkeling van hartfalen bij mensen.

6. Cardiale cTnI degradatie heeft geen invloed op β-adrenerge en voorbelasting-afhankelijke responsen in humaan hartspierweefsel.

PKA-gemedieerde desensitisatie, evenals de sarcomeerlengte-afhankelijke toename van de kracht-genererende capaciteit de myofilamenten was onveranderd in hartspiercellen met $cTnI_{1-192}$. Dit wijst erop dat de respons op cardiovasculaire stress (zoals bijvoorbeeld tijdens lichamelijke inspanning) niet verminderd zal zijn in humane harten die gedegradeerd cTnI bevatten.

Kort samengevat wordt in dit proefschrift beschreven in welke mate MZKs de energetische eigenschappen van humaan hartweefsel beïnvloeden, en wordt aangetoond dat cTnI van grote invloed is op de Ca^{2+}-gevoeligheid.

Chapter 8

Короткий зміст і висновки

Короткий зміст

Під час серцевої недостатності в серці людини відбуваються зміни в скоротливих білках. Вони включають як зміни в синтезі білків (трансляційні модифікації), так і регуляцію фосфорилюванням і деградацією білків (пост-трансляційні модифікації). Ці зміни можуть мати сприятливий характер і підтримувати скоротливу функцію хворого міокарду. Вони можуть бути також несприятливими і в значній мірі сприяти розвитку скоротливої дисфункції хворого серця людини. Таким чином нові терапевтичні засоби мають приймати до уваги функціональні наслідки компенсаційних і/або згубних змін в скоротливих білках, що відбуваються під час серцевої недостатності. Тому дуже важливо дослідити зміни в скоротливих білках під час серцевої недостатності у людини, а також їх вплив на функціональні властивості людської серцевої тканини.

В даній дисертації механічні і енергетичні наслідки змін в скоротливих білках, що відбуваються під час серцевої недостатності, були досліджені в здоровій і хворій тканині міокарду людини. Для того, щоб визначити ефект зміни складу ізоформ важких ланцюгів міозину (ВЛМ; myosin heavy chain, MHC), розвиток ізометричної сили і споживання АТФ при різних концентраціях кальцію були виміряні в хімічно скінованих трабекулах передсердя і м'язових смужках шлуночка. Наслідки деградації серцевого тропоніну I (cTнI; cTnI) для розвитку ізометричної сили та кінетики активації і розслаблення напруження були досліджені використовуючи серцеві міофібрили і міоцити. Модуляція складу тропоніну в людських серцевих препаратах була досягнена заміною ендогенного тропонінового комплексу екзогенним тропоніновим комплексом, що містив усічений cTнI. Склад ізоформ скоротливих білків був визначений за допомогою одновимірного содіумдодецил сульфат (СДС) - гель електрофорезу, в той час як стан фосфорилювання білків був визначений Вестерн іммуноблотінгом використовуючи спеціальні фосфо-антитіла. Для того, щоб визначити якість та міру сприятливості змін в білках для скоротливої функції серця людини склад білків був співвіднесений з функціональними властивостями людської серцевої тканини.

В **Главі 1** представлено загальний вступ і методи, використані в даній роботі. Визначено цілі дослідження.

В **Главі 2** розглянуто взаємовідносини між скоротливими властивостями і складом ізоформ ВЛМ у здоровому міокарді людини. За допомогою одновимірного СДС-поліакріламідного гель електрофорезу (СДС-ПААГ) було визначено, що в тканині шлуночка переважає ізоформа β-ВЛМ, в той час як в тканині передсердя було знайдено мінливу суміш ізоформ α- і β-ВЛМ. Розвиток сили в тканині шлуночка був в 5 разів економніший, але в 9 разів повільніший, ніж у тканині передсердя. Було знайдено суттєві лінійні кореляції між вмістом ізоформ ВЛМ, споживанням АТФ і швидкістю повторного розвитку сили. Дані кореляції вказують на те, що підвищена економія і знижена швидкість скорочення в тканині шлуночка в порівнянні з тканиною передсердя, можуть бути викликані переважанням в цій тканині ізоформи β-ВЛМ.

Швидкість споживання АТФ у препаратах, що містили "чистий" (100%) α-ВЛМ, був приблизно в 5 разів вище, ніж швидкість у препаратах, що містили "чистий" β-ВЛМ (0.181±0.018 і 0.039±0.014 ммоль·л$^{-1}$·с$^{-1}$, відповідно, для екстрапольованих значень ± їх стандартні похибки). K_{TP} (швидкість повторного розвитку сили), що відповідає "чистому" α-ВЛМ і розраховується з лінії регресії (10.8 с$^{-1}$) була значно вища, ніж відповідна величина отримана для тканини шлуночка (0.9 с$^{-1}$). Ці результати вказують на те, що навіть невеликий зсув у вмісті ізоформ ВЛМ може мати значний вплив на роботу серця людини.

За допомогою гістохімічного аналізу було визначено, що середня густина міофібрилярної тканини була значно нижча в препаратах передсердя, ніж в препаратах шлуночка (50±9 і 89±2%, відповідно). Різниця у вмісті міжклітинного простору між тканинами передсердя (20±4%) і шлуночка (11±2%) не досягла статистичної значимості (P=0.09). Корекція даних щодо пропорції міофібрилярної тканини усунула різницю в максимальній силі, підвищила різницю в АТФазній активності і незначно збільшила відносну різницю між екстрапольованими значеннями АТФазної активності "чистих" ізоформ. Вона також незначно покращила кореляцію між АТФазною активністю і вмістом β-ВЛМ. Різниця в середній густині міофібрилярної тканини між препаратами передсердя і шлуночка, а також інтра-трабекулярна мінливість передсердя мають бути взяті до уваги в дослідженнях, де використовуються ці препарати.

В **Главі 3** розглянуто взаємовідносини між функціональними властивостями і складом ізоформ ВЛМ у хворому міокарді людини. В порівнянні зі складом ізоформ ВЛМ у тканині пацієнтів з регулярним серцевим ритмом, хронічна аритмія передсердя супроводжувалася значним зсувом від швидкої α-ВЛМ до повільної β-ВЛМ (вміст β-ВЛМ був підвищений з 24.6±3.2 до 38.1±5.7% від загального вмісту ВЛМ). З іншого боку, як донорська, так і вражена термінально тканина шлуночка, містила майже виключно ізоформу β-ВЛМ: лише в двох донорських і одному хворому серцевому матеріалі було виявлено ізоформу α-ВЛМ. Економія скорочення і інші функціональні властивості були незмінні під час аритмії передсердя і при серцевій недостатності на заключній стадії. Однак, чутливість споживання АТФ до Ca^{2+} була значно вища при аритмії передсердя, ніж при регулярному ритмі серця, в той час як чутливість розвитку сили до Ca^{2+} не відрізнялася у цих препаратах. Це означає, що витрати на розвиток сили скорочення передсердя (споживання АТФ поділене на ізометричну силу) у пацієнтів з аритмією передсердя зменшуються при підвищенні концентрації Ca^{2+}. У хворій тканині шлуночка чутливість як сили, так і споживання АТФ до Ca^{2+} була підвищена в порівнянні зі здоровою донорською тканиною.

Підвищений вміст β-ВЛМ в здоровій і хворій серцевій тканині корелював зі зниженням в споживанні АТФ і в витратах на розвиток сили. На основі цих кореляцій можна зробити висновок, що зсув в складі ВЛМ в напрямку збільшення вмісту ізоформи β-ВЛМ може бути сприятливим в умовах патології тому, що менше енергії потребується для підтримки функції скорочення серця. Оскільки тканина шлуночка людини містить переважно β-ВЛМ, цей зсув є менш важливим в шлуночках, однак є видатною властивістю хворого передсердя.

За допомогою гістохімічного аналізу було визначено, що вміст міжклітинного простору (переважно зайнятий з'єднувальною тканиною) у здоровій тканині передсердь і шлуночка був менший, ніж у хворій тканині. Таким чином розвиток фіброзу може також сприяти зниженню скоротливої функції хворого міокарду людини (Глава 1, Рисунок 1.1).

В **Главі 4** описано функціональні наслідки деградації сТнІ для серцевої тканини людини. Ендогенний сТнІ було замінено на перший продукт деградації сТнІ

($cTnI_{1-192}$) або на цілий cTnI в серцевих міофібрилах і кардіоміоцитах. В присутності усіченого cTnI максимальна сила була незмінна, в той час як швидкості активації та повторного розвитку сили, а також кінетика розслаблення були сповільнені. В порівнянні із кардіоміоцитами з цілим cTnI, у препаратах, що містили комплекс $cTnI_{1-192}$, чутливість скоротливого апарату до Ca^{2+} була підвищена. Дані результати свідчать про те, що деградація cTnI в людському міокарді в основному погіршує функцію діастоли.

В **Главі 5** розглянуто вплив деградації cTnI на здатність міофіламентів реагувати на β-адренергічну стимуляцію і підвищене попереднє навантаження (ефект Франка-Старлінга) в людських кардіоміоцитах. Протеїн кіназа А (ПКА)-опосередкована десенсибілізація і залежне від довжини саркоміру підвищення здатності міофіламентів генерувати силу були збереженні в $cTnI_{1-192}$ кардіоміоцитах. Це вказує на те, що деградація cTnI не впливає на β-адренергічні і залежні від попереднього навантаження реакції в міокарді людини.

Висновки

1. Скорочення міокарду людини в 5 разів економніше в тканині шлуночка, ніж в тканині передсердя.

П'ятиразова різниця в економії скорочення є результатом зменшеного споживання АТФ тканиною шлуночка, оскільки ніякої суттєвої різниці не було знайдено між препаратами шлуночка і передсердя у величині відношення ізометричної сили до площі поперечного розрізу. Ця функціональна відмінність має бути взята до уваги при використанні результатів, отриманих в тканині шлуночка, для того, щоб робити висновки про властивості тканини передсердя, і навпаки.

2. Ізоформа β-ВЛМ людини в 5 разів економніша, однак значно повільніша ніж ізоформа α-ВЛМ.

З кореляцій між швидкістю споживання АТФ і вмістом ВЛМ може бути розрахована швидкість споживання волокон з "чистою" ізоформою α-ВЛМ і "чистою" ізоформою β-ВЛМ. Зменшення значення витрат на розвиток сили волокон з 100% -ним вмістом ізоформи β-ВЛМ є результатом зменшення швидкості споживання АТФ, оскільки не

було знайдено жодної кореляції між відношенням ізометричної сили до площі поперечного розрізу і вмістом ізоформи β-ВЛМ.

3. В умовах патології зсув у вмісті ВЛМ в напрямку ізоформи β-ВЛМ може бути сприятливим в тканині передсердя людини, однак в тканині шлуночка відіграє незначну роль або жодної ролі.

Оскільки ізоформа β-ВЛМ економніша ніж α-ВЛМ, підвищений вміст β-ВЛМ в серцевих м'язових волокнах буде сприятливим для хворого серця людини, оскільки менше енергії буде потребуватися для підтримки його скоротливої функції. Однак, треба зазначити, що при значному серцево-судинному стресі зсув ізоформ ВЛМ може стати й несприятливим. Серцевий резерв, необхідний в цих умовах, значною мірою визначається швидкістю скорочення міокарду, а вона буде значно сповільнена зі зростанням вмісту β-ВЛМ. Дані ефекти характерні переважно для тканини передсердя, оскільки здорова тканина передсердя містить лише 25% β-ВЛМ. Тому зсув ізоформ ВЛМ, що спостерігався під час захворювання серця (наприклад, аритмії передсердя), буде мати значні функціональні наслідки. Тканина шлуночка людини переважно містить β-ВЛМ (аж до 100%). Відповідно, зсув ізоформ ВЛМ, що може відбутися у деяких людей, буде значно меншим ніж в передсерді і тому матиме менший вплив на скоротливу функцію міокарду.

4. Безпосередній вплив змін складу тропоніну на функціональні властивості серцевої тканини людини може бути визначений заміною модифікованого тропонінового комплексу в препарати серця людини.

Як було показано за допомогою цифрової мікроскопії, протокол, використаний для заміни тропонінового комплексу в людські кардіоміоцити, забезпечує його однорідну заміну в кардіоміоцитах. За даних умов замінені кардіоміоцити розвивають ізометричну силу, порівняну з не заміненими кардіоміоцитами, і залишаються стабільними під час нанесення усього ряду субмаксимальних кальцієвих активації. Це робить заміну чудовим засобом для вивчення специфічних наслідків як трансляційних, так і пост-трансляційних модифікацій субодиниць серцевого тропоніну в серцевій тканині людини.

5. Деградація cТнI з його С-кінця може сприяти діастоличній дисфункції в міокарді людини.

Після заміни тропонінового комплексу в людських серцевих міофібрилах і кардіоміоцитах на тропоніновий комплекс з $cTnI_{1-192}$, максимальна сила була незмінна, що вказує на те, що деградація cТнI не впливає на функцію систоли. Сповільнення кінетик розслаблення і активації і підвищена чутливість до Ca^{2+} скоротливого апарату в препаратах, що містили $cTnI_{1-192}$, свідчить про те, що деградація cТнI може скоріше погіршувати функцію діастоли і таким чином сприяти розвитку серцевої недостатності у людей.

6. Деградація серцевого ТнI не впливає на β-адренергічні і залежні від попереднього навантаження реакції в міокарді людини.

ПКА- опосередкована десенсибілізація і залежне від довжини саркоміру підвищення здатності міофіламентів генерувати силу були збереженні в $cTnI_{1-192}$ кардіоміоцитах. Це вказує на те, що реакції на серцево-судинний стрес (наприклад, під час фізичних вправ) не будуть притуплені в людських серцях, що містять деградований cТнI.

Підсумовуючи, в цій дисертації було встановлено до якої міри ВЛМ визначають енергетичні властивості серцевої тканини людини і показано, що cТнI відіграє головну роль у визначенні її чутливості до Ca^{2+}.

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Curriculum Vitae

Nadiya Anatoliyivna Narolska was born on September 25th 1979 in Kiev, Ukraine. From 1986 until 1994, she attended primary and secondary schools # 63 and # 61 in Kiev. In 1994 she entered the College 'Technical lyceum of the Ukrainian National Technical University' and graduated in 1996 with honour. She started to study biology (specialization biophysics) at the Taras Shevchenko Kiev National University, Ukraine in 1996 and obtained her Master degree with honour in 2001. She conducted her graduation scientific Master's work in the Department of Biophysics, Peter Bogach Physiology Research Institute, Taras Shevchenko Kyiv National University, under supervision of Prof.Dr. Valentina M. Danilova. The subject of this work was: "Investigation of physical, chemical and functional properties of proteins of the troponin complex of the myocardium". In October 2001 she started to work on her PhD project in the same group. From February until April 2002 she worked on a collaborative project in the Laboratory for Physiology, Faculty of Medicine, Vrije Universiteit Amsterdam in the group of Dr. Ger Stienen and Dr. Jolanda van der Velden. From 2002 until 2006 she was a PhD student in the same group, under supervision of her promoter, Prof.Dr. Geert Jan Tangelder. During this period she conducted the studies on contractile protein alterations in human heart failure presented in this thesis. In 2004 she worked on a collaborative project in the Research Laboratory of Molecular Cardiology, Bergmannsheil/ St. Josef-Hospital, Medical School of the Ruhr-University of Bochum, Germany, under supervision of Prof.Dr. Kornelia Jaquet. Later the same year, she spent 2 months working in the group of Prof.Dr. Corrado Poggesi in Dipartimento di Scienze Fisiologiche, Universita di Firenze, Italy.